Expression patterns of the circadian proteins PER2 and BMAL1 throughout the rodent forebrain: immunofluorescent mapping of their co-expression with Enkephalin and Substance P

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ABSTRACT

Expression patterns of the circadian proteins PER2 and BMAL1 throughout the rodent forebrain: immunofluorescent mapping of their co-expression with Enkephalin and Substance P

Ariana Frederick

Despite rhythmic clock gene expression being found throughout the central nervous system, very little is known about their function outside of the suprachiasmatic nucleus. Understanding how clock genes are expressed across a variety of neural cell types is important to elucidating how these clock genes are regulated and how they may influence the function of each brain region. Using immunofluorescence and confocal microscopy, we quantified the co-expression of the clock proteins BMAL1 and PER2 with Substance P (SubP) and Enkephalin (Enk). Regions examined included the limbic forebrain (the dorsal striatum, ventral striatum, amygdala, stria terminalis), the thalamus (medial habenula), the hypothalamus (paraventricular nucleus, arcuate nucleus) and the olfactory bulb. In most regions examined, PER2 and BMAL1 were homogenously expressed in nearly all cells (~90%), despite very different expression profiles of SubP or Enk in each nucleus. In nuclei that expressed both SubP and Enk, PER2 and BMAL1 were not preferentially co-expressed in one cell type or the other. The olfactory bulb was unique and expressed PER2 or BMAL1 in a much smaller percentage of cells, and Enk was rarely found in the same cells that expressed the clock proteins (SubP was undetectable). This indicates that clock genes are not unique to specific cell types and further studies will be required to address which factors contribute to clock gene rhythmicity. A focus on network interactions is recommended in order to determine how different cell types contribute to various aspects of rhythmicity, such as rhythm generation, amplitude and synchrony.

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Arc	Arcuate nucleus
BLA	Basolateral nucleus of the amygdala
Bmal1, BMAL1	Brain and muscle Arnt-like *gene*, protein 1
BNSTov	Bed nucleus of the stria terminalis, oval nucleus
BNSTp	Bed nucleus of the stria terminalis, principal nucleus
cAMP	Cyclic adenosine monophosphate
CEA	Central nucleus of the amygdala
Clock, CLOCK	Circadian Locomotor Output Cycles Kaput *gene*, protein
CRE	cAMP response element
CREB	cAMP response element-binding protein
CRH	Corticotropin-releasing hormone
Cry, CRY	Cryptochrome *gene*, protein
Enk	Enkephalin
GABA	Gamma-Aminobutyric acid
HabL	Habenula, lateral
HabM	Habenula, medial
Hip	Hippocampus
i.p.	intraperitoneal
NAcC	Nucleus Accumbens, core
NAcSh	Nucleus Accumbens, shell
NDS	Normal donkey serum
NMDA	N-methyl-D-aspartate
NPY/AgRP	Neuropeptide Y and Agouti-related peptide
OB	Olfactory bulb
PBS	Phosphate buffered saline
PBS-TX	Triton-X in phosphate buffered saline
Per, PER	Period *gene*, protein
PKA	Protein kinase A
POMC	Pro-opiomelanocortin
PVH	Paraventricular nucleus of the hypothalamus
PVT	Paraventricular nucleus of the thalamus

SCN	Suprachiasmatic nucleus
SEM	Standard error of the mean
SNr	Substantia nigra, pars reticulata
SPZ	Subparaventricular zone
StrL	Striatum, lateral
StrM	Striatum, medial
SubP	Substance P
TRH	Thyrotropin releasing hormone
Tub	Olfactory tubercle
VTA	Ventral tegmental area

INTRODUCTION

Theoretical context and "The big picture"

Circadian rhythms are essential to most organisms, helping them adapt to, and survive within their environment by influencing behaviour and physiology in a 24-hour manner. In mammals, the genes at the core of our circadian system feed into the genetic control of processes as widespread as hormonal regulation, cardiac function, autonomic regulation, metabolism, arousal, memory, and sleep architecture, so it is not surprising that circadian disruption has been associated with multiple health-related risk factors (Takahashi et al., 2008; Buhr and Takahashi, 2013; Silver and Kriegsfeld, 2014; Smarr et al., 2014; Zelinski et al., 2014; Webb et al., 2015). For example, frequent shift work across a career has been linked to increased risk of affective disorders, cancer, diabetes and coronary problems (Zelinski et al., 2014).

While the link with physiological conditions can be explained by circadian regulation of hormones, metabolism, cell proliferation and tumor suppression (Albrecht et al., 2007; Takahashi et al., 2008; Zelinski et al., 2014), how circadian genes interact with neuropsychology and drive behaviour still needs to be elucidated. Sleep and circadian disruptions are well-documented symptoms of both psychological and neurodegenerative diseases (Wulff et al., 2010; McCarthy and Welsh, 2012; Videnovic and Golombek, 2013; Breen et al., 2014; Videnovic et al., 2014), but whether circadian disruption is a result of an altered neurophysiological state, or a cause, is not clear. Major depressive disorder, bipolar disorder and seasonal affective disorder have been associated with polymorphisms in clock genes (McClung, 2007; Lavebratt et al., 2010a; Lavebratt et al., 2010b; McCarthy and Welsh, 2012). On the other hand, forced circadian disruption, such as rotating shift work, is linked with worsened age-related cognitive decline, increased severity of affective disorder symptoms and memory deficits (McCarthy and Welsh, 2012; Zelinski et al., 2014), so the relationship is likely bi-directional.

The interaction between circadian clock genes and neural function is important for understanding normal behaviour and disease development and may provide potential targets for therapeutics. The first aim of this project was to anatomically determine the interaction between core clock proteins and two neuropeptides with comparatively different expression patterns throughout the rodent forebrain in order to determine if the expression of the proteins at the core

of circadian control are unique to specific cell types in the brain. A secondary aim of this project was to specifically explore the relationship between clock genes and the dopaminergic pathways that modulate motivation, reward and motor control. Dopamine has been implicated in many of the neural conditions also linked with circadian disruption and has been found to play a role in regulating clock gene rhythms (Yujnovsky et al., 2006; Imbesi et al., 2009; Hood et al., 2010; Gravotta et al., 2011). Therefore, the neuropeptides used here were specifically chosen as markers of dopaminergic signaling in the striatum, such that specific interactions found here, with known cell types, could shed light on factors that contribute to rhythmicity of clock genes in this region.

The clock genes that drive circadian rhythms

Circadian rhythms are endogenous to nearly every organism, persisting in the absence of the daily cues they are associated to. For example, rodents are active at night and sleep during the day, but remove the light and dark phases associated to "day" and "night" and the animal will continue to demonstrate a sleep-wake cycle closely similar to a regular 24-hour day. The molecular clockwork responsible for driving and maintaining these persistent rhythms consists of a series of interlocking transcription-translation negative feedback loops (Takahashi et al., 2008; Colwell, 2011; Buhr and Takahashi, 2013). In its simplest form, the core transcriptional loop involves the genes Circadian Locomotor Output Cycles Kaput (*Clock*), Brain and muscle Arnt-like gene I (*Bmal1)*, Cryptochrome (*Cry*) and Period (*Per*). CLOCK and BMAL1 make up the positive limb, forming a heterodimer that binds to the E-box of the *Cry* and *Per* genes, promoting their transcription. Their protein products, CRY and PER, associate to form the negative limb of the feedback loop by preventing the promoting effects of CLOCK and BMAL1. The degradation of CRY and PER over 24 hours eventually allows the cycle to restart.

The output of clock genes occurs at multiple levels. It is estimated that approximately 10% of the mammalian genome is regulated in a circadian manner (Lowrey and Takahashi, 2004). This regulation can occur directly, where clock proteins act as promoters to other genes, or indirectly, by regulating the promoters of downstream target genes. The ensuing effects lead to alterations in body temperature, hormone production, metabolic activity and neural firing, among many others, which can cause further signalling cascades to respond in a circadian manner.

Neural control of circadian rhythms

Circadian clock genes in mammals can be found in most tissues throughout the body and contribute to the normal functioning of each tissue type. However, a small nucleus in the ventral hypothalamus, called the suprachiasmatic nucleus (SCN), is considered to be the master controller (Dibner, 2009). The SCN receives direct input from the ganglion cells of the retina. These retinal photoreceptors are polarized by light and increase firing in the SCN. This leads to membrane changes that activate internal signaling cascades, which in turn modulate core clock genes; allowing the phase of the molecular clock to be reset according to the environmental lighting conditions (Colwell, 2011; Silver and Kriegsfeld, 2014). This allows the integration of day-night and allows the animal to align its rhythms to the 24-hour day.

Circadian rhythm control in the brain is hierarchical; the SCN acts as the "master controller" to synchronize clock gene rhythms throughout the body, communicating to other brain regions and to peripheral tissues synaptically and through secreted hormones and peptides (Dibner et al., 2010; Colwell, 2011). Most of the direct neural outputs of the SCN project to other regions of the hypothalamus and to the thalamus (Guilding and Piggins, 2007; Dibner et al., 2010). However, clock gene rhythms have been reported extensively throughout the brain (Namihira et al., 1999; Masubuchi et al., 2000; Asai et al., 2001; Shieh, 2003; Harbour et al., 2013). Rhythmicity of an animal is most commonly assessed by measuring locomotor activity, but body temperature, melatonin levels and circulating cortisol are also considered to be directly under control of the SCN. The signals from the SCN are robust and removal of this nucleus leads to abolishment of most physiological and behavioural rhythms (Mistlberger, 2005; Silver and Kriegsfeld, 2014).

The role of the SCN in circadian rhythm control is currently well understood, but we are only just beginning to understand the role of clock genes in other brain areas. Control of the sleep-wake cycle and feeding behaviour have a strong neural basis of control, and have been linked to clock gene function in a number of brain regions (Mistlberger, 2005; Verwey and Amir, 2009; Smit et al., 2013). There is also evidence that clock genes contribute to changes in mood, motivation, arousal, attention, and memory, along with many other behavioural alterations (Gerstner et al., 2009; Goel et al., 2013; Rawashdeh et al., 2014; Smarr et al., 2014). It is proposed that clock genes in these downstream brain regions respond to diverse stimuli and play

a more minor role in fine-tuning the timing of daily behaviour to relevant stimuli such as availability of food, potential mates and reward signals (including drug cues), however still very little is known about the factors that contribute to rhythmicity in these downstream regions, or exactly how they influence behavioural changes.

Dopaminergic regulation of clock genes

A number of neurotransmitter systems, including dopamine, are regulated in a 24-hour manner (Kafka et al., 1986a; Kafka et al., 1986b; Castaneda et al., 2004) and many dopamine-related behaviours fluctuate throughout the day, such as drug seeking, sensitivity to drugs of abuse, locomotor activity and motivation (Mendoza and Challet, 2014; Webb et al., 2015). Similarly, numerous aspects of dopamine signalling have also been found to be under circadian control. This includes dopamine synthesis (as reported by levels of tyrosine hydroxylase), levels of metabolites, activity of transporters and availability in the extracellular milieu (Owasoyo et al., 1979; Kafka et al., 1986a; Sleipness et al., 2007; Cai et al., 2010; Ferris et al., 2014). Extracellular dopamine levels in the rodent striatum are highest in the dark phase, when rodents are most active (Owasoyo et al., 1979; Smith et al., 1992; Paulson and Robinson, 1994; Hood et al., 2010), which has been contributed to changes in the activity of dopamine transporters that allow re-uptake into the dendritic terminal (Ferris et al., 2014), but also corresponds to increased activation patterns in the ventral tegmental area (VTA), the area where many dopaminergic cells originate. This is when the greatest number of VTA neurons expressing tyrosine hydroxylase also co-express *cFos* (Baltazar et al., 2013), and when an increase in the number of dopaminergic neuron firing occurs (Domínguez-López et al., 2014). This also appears to parallel changes to electrophysiological parameters in response to a dopamine antagonist, where local field potential oscillation frequency and coherence are altered by time of day (Frederick et al., 2014).

As is frequently the case within the circadian system, the relationship between circadian rhythms and dopamine seems to be bi-directional. The dopaminergic system appears to be directly under control of clock genes. Mutation or knockout of different circadian clock genes leads to a decrease in dopamine binding to D2 receptors (Shumay et al., 2012), an increase in depressive and anxiety-like behaviours (Lavebratt et al., 2010a; Lavebratt et al., 2010b; Mukherjee et al., 2010; Spencer et al., 2013), increased susceptibility for addiction (Shumay et al., 2012) and an altered response to drugs that act upon the dopaminergic system (Abarca et al.,

2002; Liu et al., 2005; McClung et al., 2005; Webb et al., 2009). In the other direction, dopaminergic signalling is important in clock gene expression patterns and circadian behaviours. This was first probed in animal models using methamphetamine and cocaine, where regular drug administration was able to re-entrain free-running activity rhythms under constant lighting conditions or following SCN lesion (Honma et al., 1987; Honma et al., 1992). Regular methamphetamine administration also causes a shift in the phase of clock gene expression patterns outside of the SCN (Masubuchi et al., 2000; Natsubori et al., 2013a; b; 2014), and cocaine administration causes an increase in PER1 and PER2 expression in the striatum and nucleus accumbens (Lynch et al., 2008; Falcon and McClung, 2009). These effects are prevented if a dopamine antagonist is given prior to the administration of the drug (Nikaido et al., 2001; Mohawk et al., 2013), pointing to a connection between dopamine signalling and extra-SCN clock gene function; and demonstrating that behavioural rhythms can be dissociated from SCN rhythms by manipulating dopaminergic pathways.

A recent project in our lab has been able to more directly manipulate circadian clock gene expression with dopaminergic manipulations. Depletion of dopamine in the forebrain impaired rhythms of PER1 and PER2 in the dorsal striatum, oval nucleus of the bed nucleus of the stria terminalis (BNSTov) and paraventricular nucleus of the hypothalamus (PVH), but not the central nucleus of the amygdala (CEA) (Hood et al., 2010; Gravotta et al., 2011). This effect was linked to D2-receptor signalling, as the results could be mirrored by constant infusion of a D2-receptor antagonist over five days (Hood et al., 2010). Once blunted, protein rhythms could be re-entrained to a D2-receptor agonist. Most notably, no effects were found using either a D1-receptor agonist or antagonist. Other groups have been able to show similar roles in dopamine control of clock genes. Mice lacking D2-receptors have lower levels of PER2 (Sahar et al., 2010) and Imbesi et al. (2009) were able to alter the timing of PER1 by altering the time of day that a D2-receptor agonist was administered. In the retina, dopamine, and more specifically D2-receptor signaling, has been shown to directly regulate the circadian clock by affecting BMAL1 stability and influencing light-induced entrainment, indicating that there might be a direct interaction between D2-receptor signaling and clock-gene regulation (Doi et al., 2006; Yujnovsky et al., 2006). Behavioural models in both primates and mice have shown that dopamine depletion in the basal forebrain leads to a loss of activity rhythms in constant lighting conditions (either constant light or constant dark) and impairs phase advances or phase delays in

response to light pulses, linking the functional role of dopamine to circadian related behaviours (Fifel and Cooper, 2014; Fifel et al., 2014).

Dopaminergic pathways and receptor function

Dopamine is a prominent neuromodulator that is produced in discrete brain areas that project extensively throughout the rest of the brain and spinal cord, contributing to diverse functions such as motor control, affect, arousal, hormonal regulation and the rewarding effects of drugs; and is implicated in conditions such as Parkinson's disease, Huntington's disease, major depression, schizophrenia, Tourette's syndrome and obsessive compulsive disorder (Beaulieu and Gainetdinov, 2011; Tritsch and Sabatini, 2012).

There are two principal dopaminergic systems in the central nervous system, the first, called the mesencephalic, or midbrain dopamine system, modulates behaviour through olfaction, emotion, motivation, arousal and locomotor activity (Albanese et al., 1986; Ikemoto, 2007). This is classically known as the "reward pathway". The three main pathways that make up the midbrain dopamine system consist of the mesocortical, mesolimbic and nigrostriatal pathways. The mesolimbic dopamine neurons are located in the VTA and project mostly to the nucleus accumbens and olfactory tubercle, whereas the mesocortical neurons send projections from the VTA throughout the limbic forebrain, including the hippocampus (Hip), amygdala, stria terminalis, lateral habenula (HabL), piriform cortex and medial prefrontal cortex. The nigrostriatal dopamine neurons are located in the substantia nigra, pars compacta and project to the dorsal striatum and other regions in the basal ganglia. The second dopaminergic system originates from the diencephalon, where dopaminergic neurons project from the PVH, preoptic area and arcuate nucleus (Arc) with their targets in the spinal cord, pituitary and other thalamic regions. These neurons regulate nociception, movement, sexual behaviour and the regulation and release of hormones such as prolactin. Dopaminergic circuits also exist in the olfactory bulb (OB) and retina where locally produced dopamine modulates olfactory and visual perception, respectively.

Dopamine receptor functions are as diverse as the behaviours they control and act to regulate the neurotransmission of gamma-aminobutyric acid (GABA) and glutamate via coupled G proteins. There are five types of dopamine receptors that have been characterized into two major classes based on their function (Beaulieu and Gainetdinov, 2011; Tritsch and Sabatini,

2012). D1-class dopamine receptors include types D1 and D5, are primarily located post-synaptically and are generally classified as excitatory; whereas D2-class dopamine receptors include types D2, D3 and D4, can be located post-synaptically or on the presynaptic dopaminergic neuron as auto-receptors, and are generally inhibitory. These receptors act to up- or down-regulate neurotransmission by altering the probability of presynaptic neurotransmitter release (usually GABA or glutamate), modifying the postsynaptic receptor sensitivity to the available neurotransmitters, or adjusting either the pre- or post-synaptic membrane excitability; thus allowing a multitude of changes to the neural circuitry. D1-receptors are coupled to $G\alpha_s$ that activates adenyl cyclase, increasing cyclic adenosine monophosphate (cAMP), which leads to the activation of protein kinase A (PKA). Most of the effects of D1-receptor signalling are carried out via PKA, which regulates various voltage-gated ion channels, ionotropic glutamatergic and GABAergic receptors and activates transcription factors in the nucleus. D2-receptors are coupled to $G\alpha_{i/o}$ that inhibits adenyl cyclase, thus preventing activation of PKA-mediated changes to the neuron (Gerfen and Surmeier, 2011; Tritsch and Sabatini, 2012). Once the receptor is activated and the Gα subunit has dissociated, the Gβ and Gγ also have the ability to diffuse across the membrane and activate ion channels and other second-messenger systems; a phenomenon more frequently seen with the D2-receptors. And finally, certain domains of the D1- and D2-receptors are able to interact directly with subunits of N-methyl-D-aspartate (NMDA) and $GABA_A$ receptors, altering their function and availability at the membrane.

The functions of the different subtypes of dopamine receptors have most thoroughly been studied in the striatum and limbic forebrain where all five sub-types of dopamine receptors are found, however D1- and D2-receptors are the most common. D1- and D2-receptors are widespread throughout the brain, particularly the limbic forebrain (hippocampus, amygdala, prefrontal cortex and piriform cortex) and hypothalamus. However differences do exist between their expression patterns (Bouthenet et al., 1987; Levey et al., 1993; Coronas et al., 1997). For example, D1-receptors are more concentrated to the basolateral nucleus of the amygdala (BLA) and are weakly expressed in the OB, whereas D2-receptors are more localized to the CEA and are highly expressed in the glomerular layer of the OB. As one could imagine, the combination of dopamine receptor types, along with the principal cell type (GABA or glutamate), the location of the dopamine receptor at the synapse and the great diversity of channels, receptors and intercellular pathways that can be influenced, makes understanding the effects of dopamine on

behaviour quite complicated and has therefore become of great interest to many behavioural neuroscientists. Given the potential for circadian rhythms to also drive behaviour, understanding how these two systems interact will provide further insights into the mechanisms of behavioural control.

Markers of dopaminergic receptors

As mentioned above, dopamine's modulatory role to the pathways of the basal ganglia have been well studied. The dorsal striatum receives excitatory inputs from nearly all regions of the cortex and some areas of the thalamus, which synapse onto inhibitory medium spiny neurons that are segregated into two separate efferent pathways (Gerfen et al., 1990; Lu et al., 1998; Steiner and Gerfen, 1998; Gerfen and Surmeier, 2011). Striatonigral medium spiny neurons project to the substantia nigra pars reticulata (SNr) (homologous to internal segment of the globus pallidus in primates) along the "direct" pathway, whereas striatopallidal medium spiny neurons project to the globus pallidus or ventral pallidum (homologous to the external segment of the globus pallidus in primates) along the "indirect" pathway. D1-receptors are predominately expressed on striatonigral neurons, while D2-receptors are concentrated on the striatopallidal neurons. Striatal medium spiny neurons can also be identified by the peptides they produce. D1-receptor bearing medium spiny neurons uniquely produce substance P (SubP) and dynorphin whereas D2-receptor bearing medium spiny neurons produce enkephalin (Enk) (Gerfen et al., 1990; Lu et al., 1998).

The nucleus accumbens is alternately called the "ventral striatum" due to its anatomic similarity with the dorsal striatum. Projections from the nucleus accumbens parallel that of the dorsal striatum, where D1-receptor bearing medium spiny neurons receiving afferents from the cortex project to the SNr and also produce SubP and dynorphin (Lu et al., 1998). Similarly, the D2-receptor bearing neurons here project to the globus pallidus and produce Enk. Some differences between the striatum and the nucleus accumbens can be noted nonetheless. First, D1- and D2-receptors are randomly distributed throughout the striatum in nearly equivalent proportions (Gerfen and Young, 1988; Gangarossa et al., 2013b), which remains to be true in the nucleus accumbens core, but they appear more compartmentalized in the shell (Gangarossa et al., 2013a). Second, the co-expression of D1- and D2-receptors is extremely low in the striatum at ~7% whereas in the nucleus accumbens this jumps to ~ 25% in the core and ~35% in the shell

(Perreault et al., 2010; Gangarossa et al., 2013a). Notably, these neurons also produce both dynorphin and Enk.

SubP, dynorphin and Enk are peptides that act as neuromodulators and neurotransmitters throughout the central nervous system and serve many purposes. In the basal ganglia, they are important to long-term changes in dopaminergic signalling and help provide negative feedback, dampening excessive excitation from increased activation of the dopaminergic inputs (Steiner and Gerfen, 1998). To the best of our knowledge, similar relationships between SubP and dynorphin with D1-receptors, and Enk with D2-receptors, has not been made outside of the basal ganglia. In the amygdala and the mediobasal hypothalamus, Enk has been linked with similar modulatory roles of dopaminergic transmission (Rotsztejn et al., 1978; Walczak et al., 1979), providing some evidence that there may be functional similarities in these other regions. Nevertheless, SubP and Enk have quite different expression patterns throughout the rodent forebrain and in this project can provide useful information regarding the extent that clock genes are expressed in different cell types in various brain regions (Gray et al., 1984; Chronwall, 1985; Everitt et al., 1986; Warden and Young, 1988).

Purpose and hypothesis

This thesis is part of a bigger aim attempting to understand the role of the circadian system in regulating behaviour and how neurotransmitter systems interact with circadian gene regulation. Dopaminergic regulation is just one example of the many possible neurotransmitters and hormones potentially regulating clock genes throughout the brain (Amir and Stewart, 2009; Ruby et al., 2014). For this reason, we intended to first answer: Are circadian clock genes expressed in specific cell types throughout the rodent forebrain? And to secondarily address: Why is PER expression specifically linked with D2-receptor and not D1-receptor signaling in the dorsal striatum, as demonstrated by Hood et al. (2010). For this project, we examined if PER2 and BMAL1 are co-expressed with SubP or Enk, markers for D1 and D2-receptor bearing neurons in the basal ganglia, but also representing specific cell-types in other regions of the brain. We expected to find a preferential co-expression between PER2 and BMAL1 with Enk in the basal ganglia, but could not predict if this relationship would be continuous throughout the brain.

Co-expression of CLOCK has previously been reported with D2-receptors in

embryonic striatal cell cultures (Imbesi et al., 2009) and with tyrosine hydroxylase in the VTA (McClung et al., 2005). However, Imbesi et al. did not address if there is also co-expression with D1-receptors, and dopamine has been shown to change its functional expression patterns in development, especially within the circadian system, so these findings cannot be generalized to the adult brain (Weaver et al., 1992; Weaver et al., 1995; Rivkees and Lachowicz, 1997). McClung et al. also only examined one cell type, in one discrete brain region, so these findings do not provide further information on the extent to which circadian genes are expressed in extra-SCN brain areas, though Figure 3a. suggests that CLOCK expression is more widespread than tyrosine hydroxylase. To elaborate these findings, we chose to examine several brain areas including the striatum, BNSTov and CEA, which responded differently to dopamine depletion (Gravotta et al., 2011), and other dopaminergic brain areas that have been shown to rhythmically express clock genes (Harbour et al., 2013) or have been shown as important outputs of the SCN (Dibner et al., 2010).

METHODS

Animals

Six male Wistar rats (Charles River, St-Constant, QC), 300-350 g in size, were used for this project. Prior to the start of the experiment, animals were housed individually under a 12h light/dark cycle for two weeks in lightproof boxes with *ad libitum* access to food and water. They also had free access to running wheels, monitored continuously using VitalView software (Mini-Mitter, Sunriver, OR). The experiment began after all animals' activity entrained to the light/dark cycle. All experimental procedures adhered to guidelines from the Canadian Council on Animal Care and were approved by the Concordia University Animal Research Ethics Committee.

Colchicine

To prevent axonal transport and enhance cell body visualization, colchicine was injected into the left lateral ventricle 24-36 h prior to perfusions (Khachaturian et al., 1983). Animals were anesthetized with a mix of ketamine and xylazine, injected intraperitoneally (i.p.), then 100

μg of colchicine (Sigma-Aldrich, Oakville, ON) dissolved into 5 μl of saline was infused at a rate of 0.5 μl/min over 10 min at the following stereotaxic coordinates: AP -3.0, ML 1.2, DV 3.6 (from the skull) (Paxinos and Watson, 1998).

Tissue preparation and immunofluorescence

In order to be able to image each brain area around the time of its peak PER2 expression, animals were perfused either one hour (ZT1) or ten hours (ZT10) after lights turned on. Rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, injected i.p.) and transcardially perfused with 300 ml of cold saline (0.9% NaCl), followed by 300 ml of cold paraformaldehyde (4% in a 0.1M phosphate buffer, pH 7.3). Brains were extracted and stored overnight in paraformaldehyde at 4°C. Four serial sets of coronal sections were collected, sliced at a thickness of 30 μm using a vibratome and then stored at -20°C in Watson's cryoprotectant (Watson et al., 1986) until ready to be used.

Double labeling was performed using the following four combinations: 1) PER2 with Enk, 2) PER2 with SubP, 3) BMAL1 with Enk and 4) BMAL1 with SubP. Only enough tissue that could be imaged within the next 5 days was processed at one time and therefore anterior and posterior sections were run at different times (stopping just posterior to the SCN, around -1.80 mm from bregma). In each run, free-floating sections were rinsed once for 10 min in phosphate buffered saline (PBS, pH 7.4), followed by 3x10 min rinses in 0.3% Triton-X in PBS (PBS-TX). Tissue was pre-blocked for 1 h at room temperature with gentle agitation in a solution of PBS-TX with 3% skim milk powder and 6% normal donkey serum (NDS) then directly transferred to the primary incubation. Tissue was incubated for 48 h with the primary antibody at 4°C with gentle agitation, rinsed 3x10 min in PBS-TX, then incubated with the secondary antibody for 1 h at room temperature with gentle agitation. Antibodies were diluted in a solution of 0.3% PBS-TX with 3% skim milk powder and 2% NDS. The following antibodies and dilutions were used: PER2 rabbit polyclonal 1:800 (Alpha Diagnostics, San Antonio, Tx) or PER2 rabbit polyclonal 1:400 (a generous gift from David R. Weaver), BMAL1 rabbit polyclonal 1:800 (Novus Biologicals, Littleton CO), Leu /Met-enkephalin [NOC1] mouse monoclonal 1:800 (Abcam, Toronto, ON), Substance P [SP-DE4-21] mouse monoclonal 1:400 (Abcam, Toronto, ON), anti-rabbit secondary Alexa-488 and anti-mouse secondary Alexa-594 1:500 (Life Technologies, Carlsbad CA)

Once all incubations were complete, the tissue was rinsed 3x10 min in PBS-TX, then treated to reduce lipofuscin-like autofluorescence with Sudan Black B (Sigma-Aldrich, Oakville, ON): 0.1% in 70% ethanol for 10 minutes (Schnell et al., 1999). The tissue was rinsed 3x10 min in PBS-TX, and a final 10 min in PBS before being mounted onto slides, allowed to air dry and coverslipped with ProLong® Diamond Antifade Mountant with DAPI (Life Technologies, Carlsbad, CA). Slides were left to cure overnight in the dark, sealed with clear nail polish and imaged over the next 5 days. While not in use, slides were stored in a slide box kept at 4°C.

Imaging and analysis

Images were captured using the 60x objective on an Olympus FV10i automated confocal laser scanning microscope at the Centre for Microscopy and Cell Imaging, Concordia University, Montreal, Canada. Brain regions of interest were determined based off of landmarks from "Brain maps: structure of the rat brain" (Swanson, 2004), with the exception of the nucleus accumbens, where landmarks were identified from "The Rat Brain In Stereotaxic Coordinates" (Paxinos and Watson, 1998). One image for each brain area was taken per slice, up to a maximum of seven slices. For most brain regions, this was sufficient to include all slices containing the area of interest. In larger areas, such as the dorsal striatum and the olfactory tubercle, slices were selected randomly and the dorsal-ventral (or medial-lateral) coordinates were shifted from image to image. Images captured an area of 212 x 212 μm, at a depth of every 0.8 μm and a quality of 1024 x 1024 pixels. Laser intensity was set automatically, and then adjusted for each brain area for optimal visualization of the fluorescent labeling.

Images were counted using ImageJ freeware (http://imagej.nih.gov). Cells in each channel were manually identified and marked layer-by-layer; each marked cell was superimposed onto every layer so as not to re-count cells that appeared in subsequent layers. A comparison with DAPI was used to define neurons based off of the distinct, large, round shape of their nuclei. A composite of the markings in each channel was created, and based off the central position of every marking we were able to extract 1) the total number of neurons as defined by DAPI-stained nuclei, 2) the number of neurons expressing either BMAL1 or PER2, 3) the number of neurons co-expressing Enk or SubP, and 4) the number of neurons expressing Enk or SubP, but not BMAL1 or PER2. These raw numbers were entered into excel for each image and converted to a percentage. The percentages were then imported into Prism (Version 6, GraphPad,

San Diego, CA) for descriptive statistics and statistical testing. Differences were determined using two-tailed, unpaired t-tests. Levels of expression for each neuropeptide were expected to be different and unique to each brain area examined. Therefore, each brain region was expected to have co-expression levels completely independent of other regions examined. For this reason, each brain area was analyzed separately, thus preventing the need for adjustments of multiple comparisons. Results presented here are displayed as the mean ± standard error of the mean (SEM). Significance was set at $p<0.05$.

RESULTS

All proteins were highly expressed in most brain regions imaged. As expected, BMAL1 and PER2 were mostly nuclear with some cytosolic expression, colocalizing very closely with the nuclear DAPI labelling. Enk and SubP were mostly cytosolic and richly expressed in fibres. Only neurons where the cell body was labeled were counted, likely making the actual count of Enk and SubP slightly higher than reported here due to interference from the labelled fibres. Nonetheless, BMAL1 and PER2 were clearly co-expressed with both Enk and SubP in brain regions containing these peptides. In fact, BMAL1 and PER2 were expressed in the majority of identified neurons in nearly all brain regions examined.

The dorsal and ventral striatum

The coexpression of PER2 and BMAL1 with Enk or SubP was first analyzed in the dorsal and ventral striatum, including the medial and lateral dorsal striatum (StrM and StrL), nucleus accumbens core and shell (NAcC and NAcSh) and olfactory tubercle (Tub). Example regions where images were taken are outlined in Figure 1. In this area of the brain Enk and SubP correspond to D2- and D1-receptor bearing medium spiny neurons respectively (Gerfen et al., 1990; Lu et al., 1998). The aim here was to explore if the circadian proteins BMAL1 and PER2 were expressed in both types of medium spiny neurons.

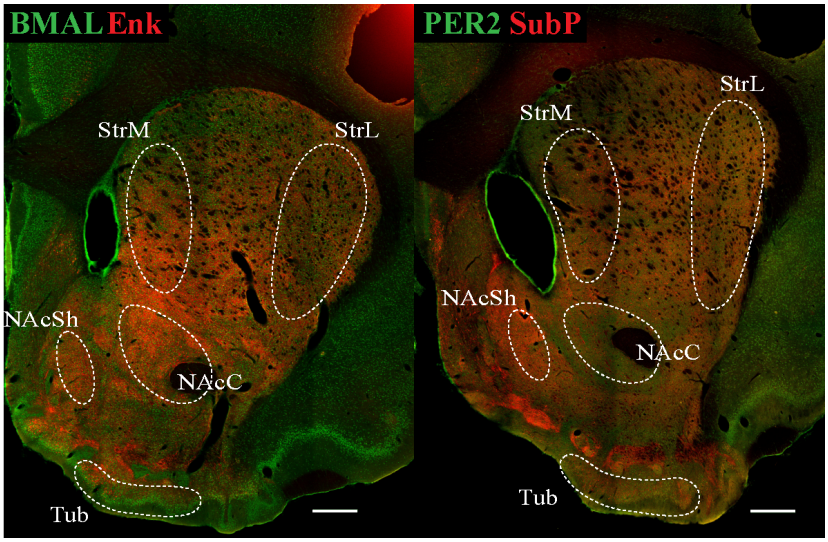

Figure 1. Outline of regions imaged in the dorsal and ventral striatum. The left panel is labelled with BMAL1 (BMAL) in green and Enkephalin (Enk) in red and the right panel is labelled with PER2 in green and Substance P (SubP) in red. Individual confocal images were stitched together to create the single image. Scale bar: 500 µm. StrM, medial dorsal striatum; StrL, lateral dorsal striatum; NAcC, nucleus accumbens core; NAcSh, nucleus accumbens shell; Tub, olfactory tubercle.

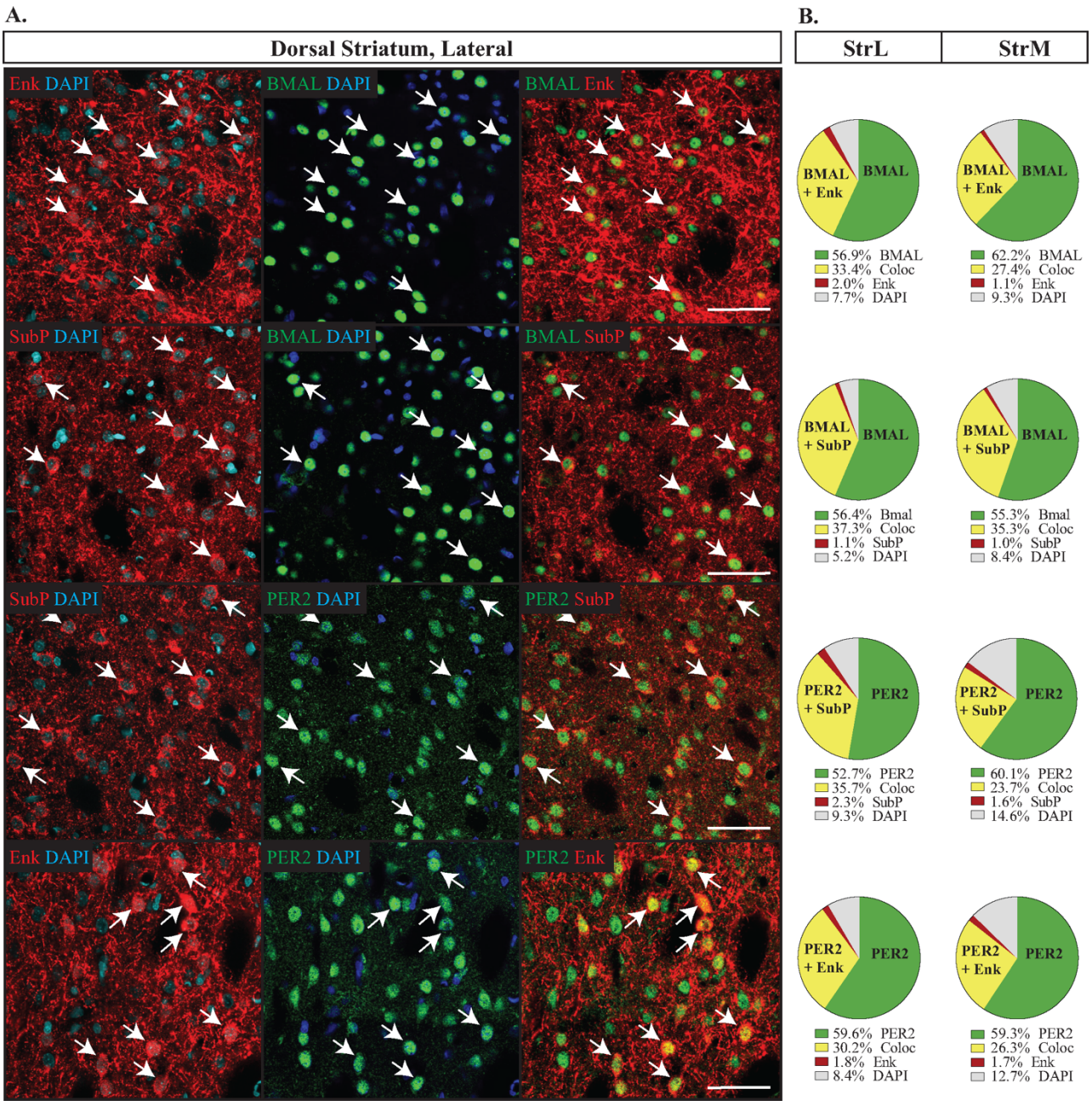

Figure 2. Localization of proteins in the dorsal striatum. (A) Each combination of antibodies analyzed are shown, sampled from the lateral dorsal striatum. In the first panel enkephalin (Enk) or Substance P (SubP) (red) is shown with DAPI (aqua). In the middle panel PER2 or BMAL1 (BMAL) (green) are shown with DAPI (blue). In the right panel BMAL or PER2 (green) are shown with Enk or SubP (red). Arrows point to the same cell in each image within each row. Scale bar: 50 µm. (B) Pie charts representing the proportion of each labelled cell for each respective combination. Green: PER2 or BMAL with DAPI only; Yellow: PER2 or BMAL1 with Enk or SubP (Coloc), representing cells with co-expression; Red: Enk or SubP with DAPI only; Grey: cells identified with DAPI but not labelled with PER2, BMAL, Enk or SubP. StrM, medial dorsal striatum; StrL, lateral dorsal striatum.

Table 1. Relative proportions of cells counted in the dorsal and ventral striatum

	BMAL ± SEM (% of total)	PER2 ± SEM (% of total)	Enk ± SEM (% of total)	SubP ± SEM (% of total)	Enk ± SEM (% of BMAL)	SubP ± SEM (% of BMAL)	Enk ± SEM (% of PER2)	SubP ± SEM (% of PER2)
StrL	92.4% ±0.7%	89.0% ±0.7%	34.7% ±1.6%	37.5% ±1.5%	39.3% ±2.5%	39.9% ±1.8%	33.5% ±1.6%	38.5% ±2.2%
	* p=0.002							
StrM	90.1% ±0.8%	85.0% ±1.2%	28.8% ±1.6%	31.3% ±1.9%	30.6% ±3.4%	39.0% ±2.3%	30.4% ±1.5%	28.3% ±2.0%
	* p=0.003							
NAcC	88.8% ±1.6%	84.9% ±1.8%	26.1% ±1.9%	26.0% ±2.4%	20.2% ±1.0%	32.7% ±2.3%	32.8% ±2.0%	26.0% ±2.7%
					* p=0.001			
NAcSh	86.7% ±1.7%	82.9% ±1.6%	22.7% ±1.9%	36.4% ±1.8%	16.3% ±1.2%	39.1% ±4.0%	30.6% ±1.3%	38.6% ±1.9%
			* p<0.001		* p<0.001		* p=0.003	
Tub	90.1% ±1.2%	82.4% ±1.7%	28.6% ±1.6%	41.1% ±2.7%	32.0% ±2.3%	37.1% ±3.4%	29.2% ±1.7%	38.8% ±2.5%
	* p=0.001		* p=0.001				* p=0.001	

Values represent the mean ± standard error (SEM). Total counts from each combination of antibodies are pooled together to get percentages of the total cells counted. Comparisons that were found significantly different are indicated with the dashed arrow, and p-values were determined by two-way unpaired t-tests. Values of $p<0.05$ are considered significant.

Table 2. Relative proportions of cells counted in the CEA, BNST, PVH, HabM, Arc & OB

	BMAL1 ± SEM (% of total)	PER2 ± SEM (% of total)	Enk ± SEM (% of total)	SubP ± SEM (% of total)	Enk ± SEM (% of BMAL)	SubP ± SEM (% of BMAL)	Enk ± SEM (% of PER2)	SubP ± SEM (% of PER2)
CEA	90.2% ±0.9%	86.7% ±0.9%	35.3% ±1.8%	8.2% ±0.7%	35.6% ±2.4%	5.9% ±1.1 %	37.5% ±2.7%	9.8% ±1.0%
	* p=0.013		* p<0.001		* p<0.001		* p<0.001	
BNSTov	91.7% ±0.8%	78.1% ±1.9%	26.7% ±1.3%	18.8% ±1.8%	25.4% ±2.5%	21.3% ±2.5 %	28.8% ±1.6%	20.3% ±3.3%
	* p<0.001		* p=0.002				* p=0.034	
BNSTp	90.5% ±2.2%	78.0% ±3.4%	-	22.3% ±1.9%	-	19.0% ±1.4 %	-	21.8% ±2.7%
PVH	83.6% ±1.6%	72.6% ±2.3%	35.2% ±1.8%	14.9% ±3.9%	29.3% ±2.5%	10.4% ±1.8 %	33.8% ±1.3%	27.3% ±7.3%
	* p=0.001		* p<0.001		* p=0.001			
HabM	71.4% ±1.4%	61.6% ±6.5%	-	52.7% ±5.8%	-	61.3% ±2.8 %	-	63.8% ±11.9%
Arc	85.5% 1.2%	78.7% 1.2%	14.5% 1.4%	15.1% 4.2%	13.3% 0.9%	7.5% 1.0%	14.5% 3.5%	27.3% 7.3%
	* p=0.010				* p<001			
OB	28.0% 3.3%	18.8% 1.3%	18.8% 0.7%	-	10.2% 1.4%	-	11.5% 1.7%	-
	* p=0.004							

Values represent the mean ± standard error (SEM). Total counts from each combination of antibodies are pooled together to get percentages of the total cells counted. The minus sign indicates no expression of this protein in the brain region. Comparisons that were found significantly different are indicated with the dashed arrow, and p-values were determined by two-way unpaired t-tests. Values of $p<0.05$ are considered significant.

Between the StrM and StrL, expression patterns of all four proteins were similar (Fig 2). Both BMAL1 and PER2 were homogenously expressed throughout the dorsal striatum, though PER2 labelled a slightly lower percentage of cells than BMAL1 (refer to Table 1 for all relative proportions and statistical differences). Enk and SubP each labelled about a third of the neurons and almost always occurred with PER2 or BMAL1. Furthermore, PER2 and BMAL1 were not preferentially co-expressed with Enk or SubP.

In the nucleus accumbens, BMAL1 and PER2 were uniformly expressed in most neurons at similar levels as the dorsal striatum. However, the expression patterns of Enk and SubP differed slightly. As reported elsewhere with D1- and D2-receptor expression (Gangarossa et al., 2013a), SubP and Enk appeared rather uniformly in the NAcC, but had a more patchy appearance in the NAcSh. Images were sampled from the medial region of the shell because it had relatively high expression of both Enk and SubP (see Fig 1). Nearly all Enk and SubP expressing cells also expressed BMAL1 or PER2 in both the core and the shell (Fig 3), however, a lower percentage of cell bodies were counted expressing Enk, leading to lower coexpression of BMAL1 and Enk in the core ($p=0.001$) and both PER2 and BMAL1 with Enk in the shell (PER2 $p=0.003$, BMAL1 $p<0.001$, Table 1). The pattern of expression from the NAcSh was extended to the Tub (Fig 4), which also had overall lower expression levels of Enk that lead to lower co-expression between PER2 and Enk.

Mesocortical projection regions (CEA, BLA, BNSTov, BNSTp, Hip)

The mesocortical dopamine system projects throughout the limbic forebrain to regions that contribute largely to behaviours including motivation, emotion and long term memory. They include the central nucleus of the amygdala (CEA) and basolateral amygdala (BLA), the oval nucleus and the principal nucleus of the bed nucleus of the stria terminalis (BNSTov and BNSTp) and the hippocampus (Hip) (Ikemoto, 2007). The CEA and BNSTov share similar anatomical and functional properties and are often referred to together as the central extended amygdala (Alheid, 2003). These two nuclei appear to be unique in their clock gene rhythm regulation. They peak in antiphase to the other limbic forebrain regions (Harbour et al., 2013), and interestingly the BNSTov, but not the CEA, is affected by global dopamine depletion (Gravotta et al., 2011), suggesting that there may be different entrainment mechanisms in these two nuclei. The two main neuronal populations of the CEA and BNSTov produce either Enk or

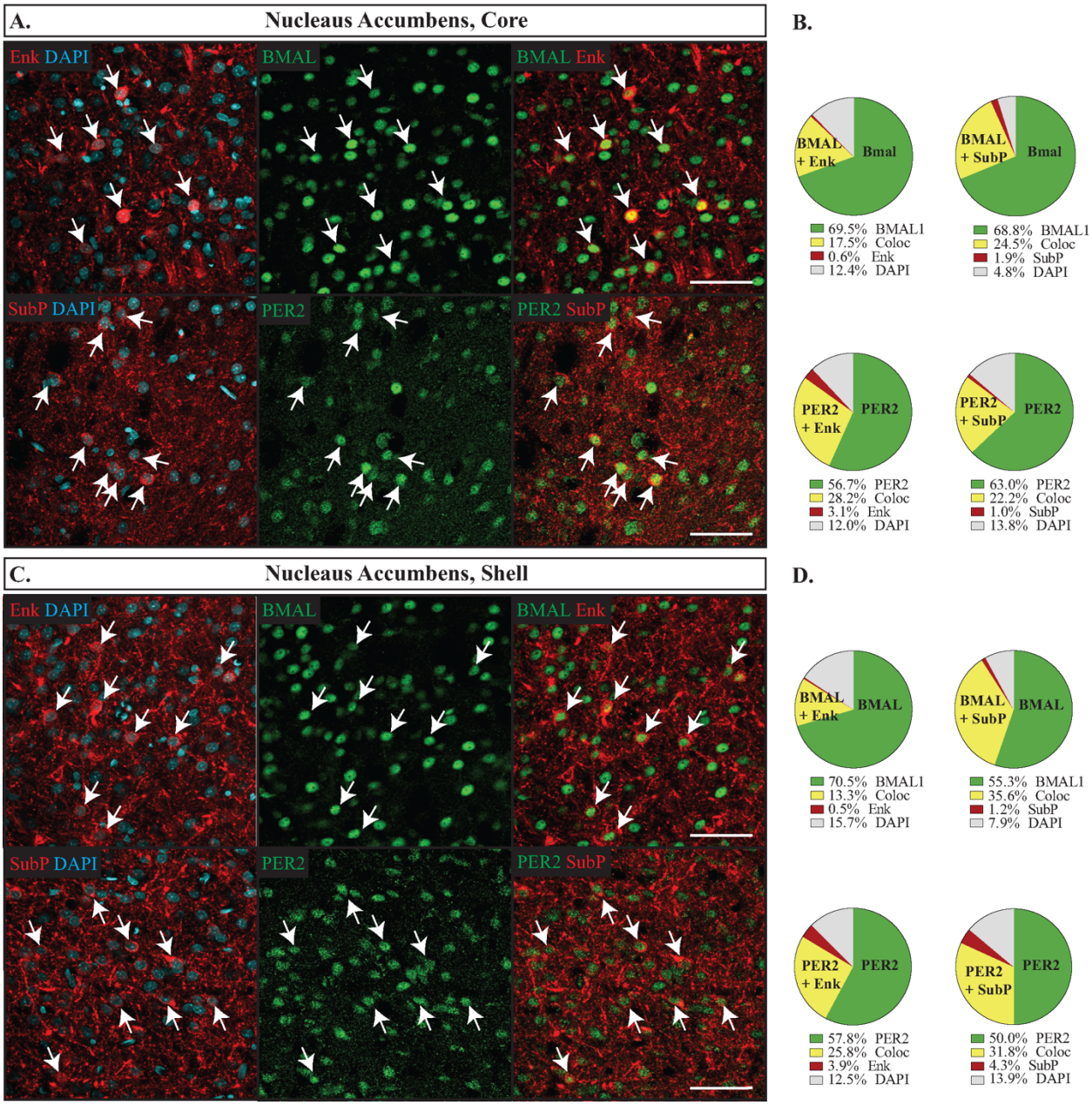

Figure 3. Localization of proteins in the nucleus accumbens. Localization of proteins in the nucleus accumbens core (A,B) and shell (C, D). Immunofluorescent images (A, C): In the first panel enkephalin (Enk) or Substance P (SubP) (red) is shown with DAPI (aqua). In the middle panel PER2 or BMAL1 (green). In the right panel BMAL1 or PER2 (green) are shown together with Enk or SubP (red). Arrows point to cells labelled with both proteins and indicate the same cell across the row. Scale bar: 50 µm. Pie charts (B, D) representing the proportion of each labelled cell combination counted in the core (B) and shell (D). Green: PER2 or BMAL1 with DAPI only, Yellow: proportion co-expressing PER2 or BMAL1 with Enk or SubP (Coloc), Red: Enk or SubP with DAPI only, Grey: cells identified with DAPI but not labelled with PER2, BMAL1, Enk or SubP.

corticotropin-releasing hormone (CRH) (Day et al., 1999), and while D2-receptors are likely found on CRH producing cells, co-expression between Enk and either type of dopamine receptor cannot be excluded (Asan, 1997).

We found that the CEA and BNSTov had similar expression patterns of Enk, but differed slightly in their expression levels of SubP. Enk labelled about a third of cells, while in both regions a significantly smaller proportion of cells were labelled with SubP, with only 8.2% ±0.7% showing immunoreactivity in the CEA and 18.8% ±1.8% in the BNSTov (Table 2). Nonetheless, Enk and SubP were nearly always co-expressed with both BMAL1 and PER2, and similar to the striatum, BMAL1 and PER2 continued to be expressed in the majority of all neurons (Fig 5 and 6). The BNSTp showed a slightly different expression profile than the BNSTov, and had nearly a third of its cells labelled with SubP but no cell bodies were found labelled with Enk. Nonetheless, BMAL1 and PER2 was still present in the majority of neurons, and SubP was consistently found with these proteins (Fig 7). In the Hip (Fig 8) and the BLA (Fig 5), Enk and SubP were only occasionally labelled (<1% of cells) and were thus not analyzed further.

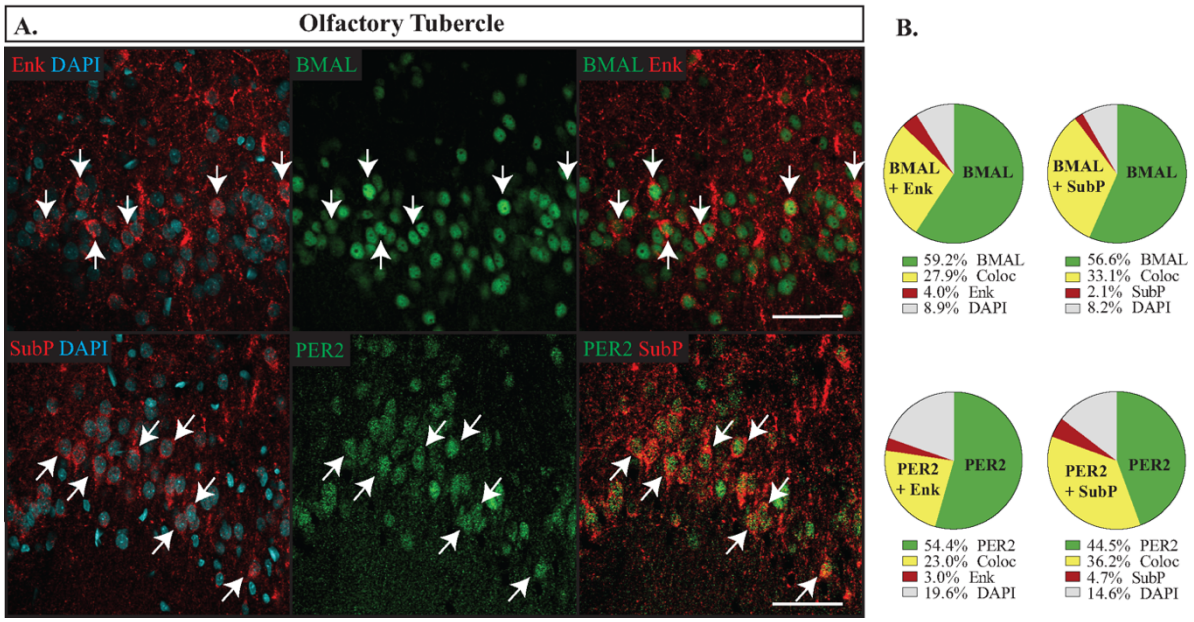

Figure 4. Localization of proteins in the olfactory tubercle. (A) Localization of proteins in the olfactory tubercle. In the first panel enkephalin (Enk) or Substance P (SubP) (red) is shown with DAPI (aqua). In the middle panel PER2 or BMAL1 (green). In the right panel BMAL1 or PER2 (green) are shown together with Enk or SubP (red). Arrows point to cells labelled with both proteins and indicate the same cell across the row. Scale bar: 50 µm. (B) Pie charts representing the proportion of each labelled cell combination. Green: PER2 or BMAL1 with DAPI only, Yellow: co-expression of PER2 or BMAL1 with Enk or SubP (Coloc), Red: Enk or SubP with DAPI only, Grey: cells identified with DAPI but not labelled with PER2, BMAL1, Enk or SubP.

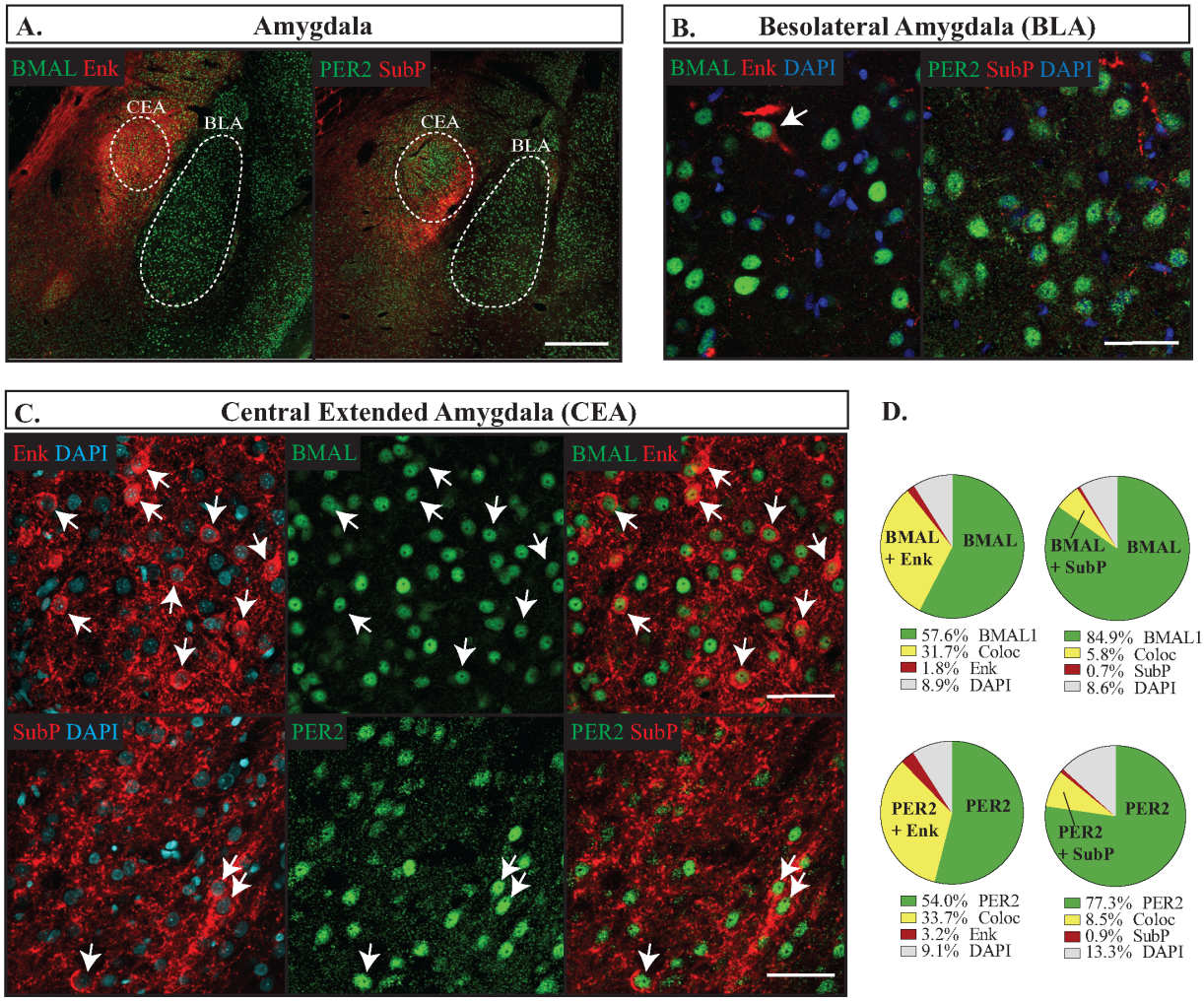

Figure 5. Localization of proteins in the amygdala. (A) Outlines of regions imaged in the central nucleus of the amygdala (CEA) and basolateral amygdala (BLA). The left panel is labelled with BMAL1(green) and Enkephalin (Enk) (red) and the right panel is labelled with PER2 (green) and Substance P (SubP) (red). Scale bar: 500 µm. Localization of proteins in the BLA (B), or the CEA (C) showing PER2 or BMAL1 (green), Enk or SubP (red), DAPI (blue). Arrows point to cells labelled with both proteins and indicate the same cell across a row. Scale bar: 50 µm. (D) Pie charts representing the proportion of cells with each labelling combination counted in the CEA. Green: PER2 or BMAL1 with DAPI only, Yellow: PER2 or BMAL1 co-expressing Enk or SubP (Coloc), Red: Enk or SubP with DAPI only, Grey: cells identified with DAPI but not labelled with PER2, BMAL1, Enk or SubP.

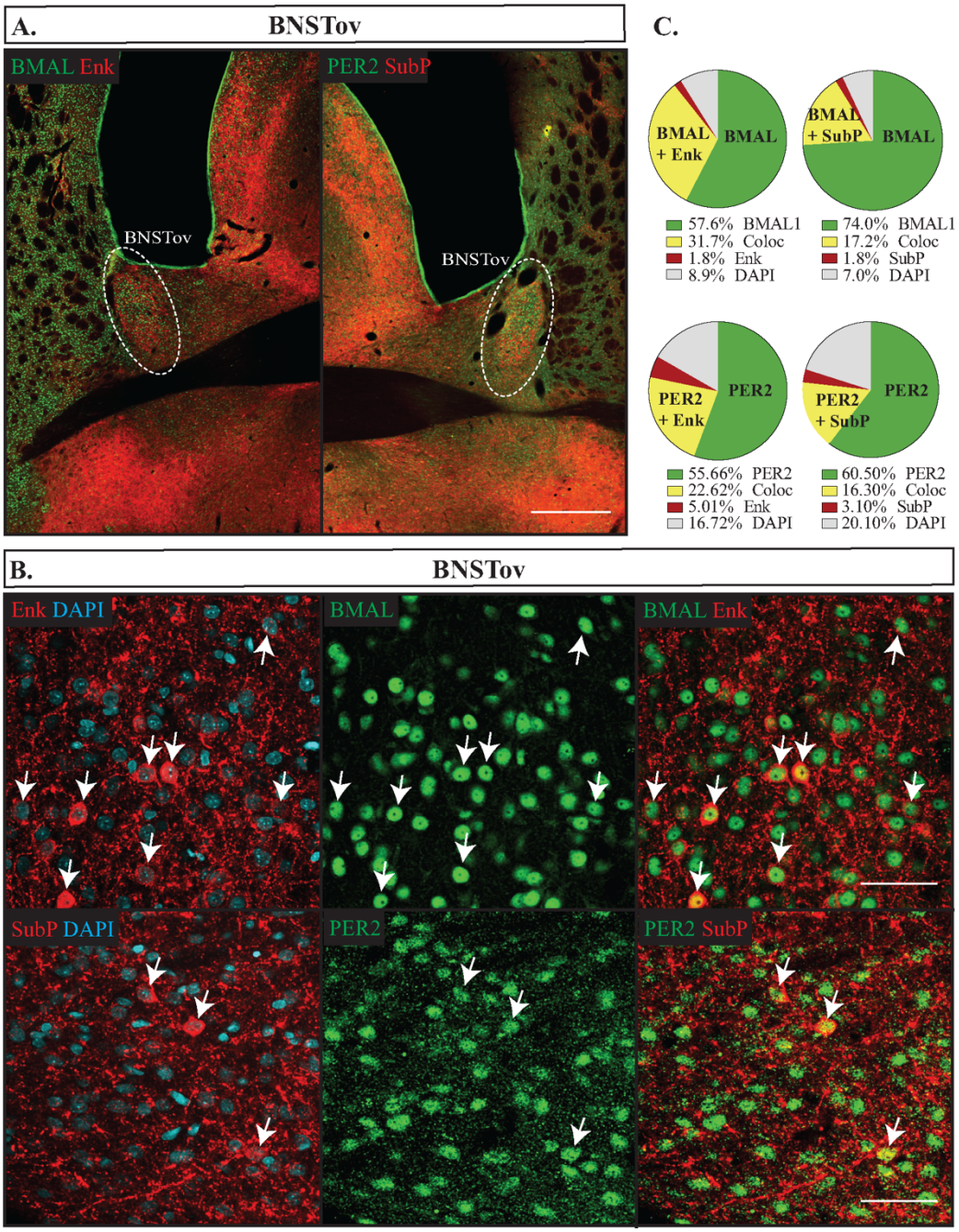

Figure 6. Localization of proteins in the BNSTov. (A) Outlines of regions imaged in the oval nucleus of the bed nucleus of the stria terminalis (BNSTov). The left panel is labelled with BMAL1 (green) and Enkephalin (Enk) (red) and the right panel is labelled with PER2 (green) and Substance P (SubP) (red). Scale bar: 500 µm. (B) Localization of proteins in the BNSTov. In the first panel Enk or SubP (red) is shown with DAPI (aqua). In the middle panel PER2 or BMAL1 (green). In the right panel BMAL1 or PER2 (green) are shown with Enk or SubP (red). Arrows point to cells labelled with both proteins and indicate the same cell across the row. Scale bar: 50 µm. (C) Pie charts representing the proportion of each labelled cell combination in the BNSTov. Green: PER2 or BMAL1 with DAPI only, Yellow: PER2 or BMAL1 co-expressing Enk or SubP (Coloc), Red: Enk or SubP with DAPI only, Grey: cells identified with DAPI but not labelled with PER2, BMAL1, Enk or SubP.

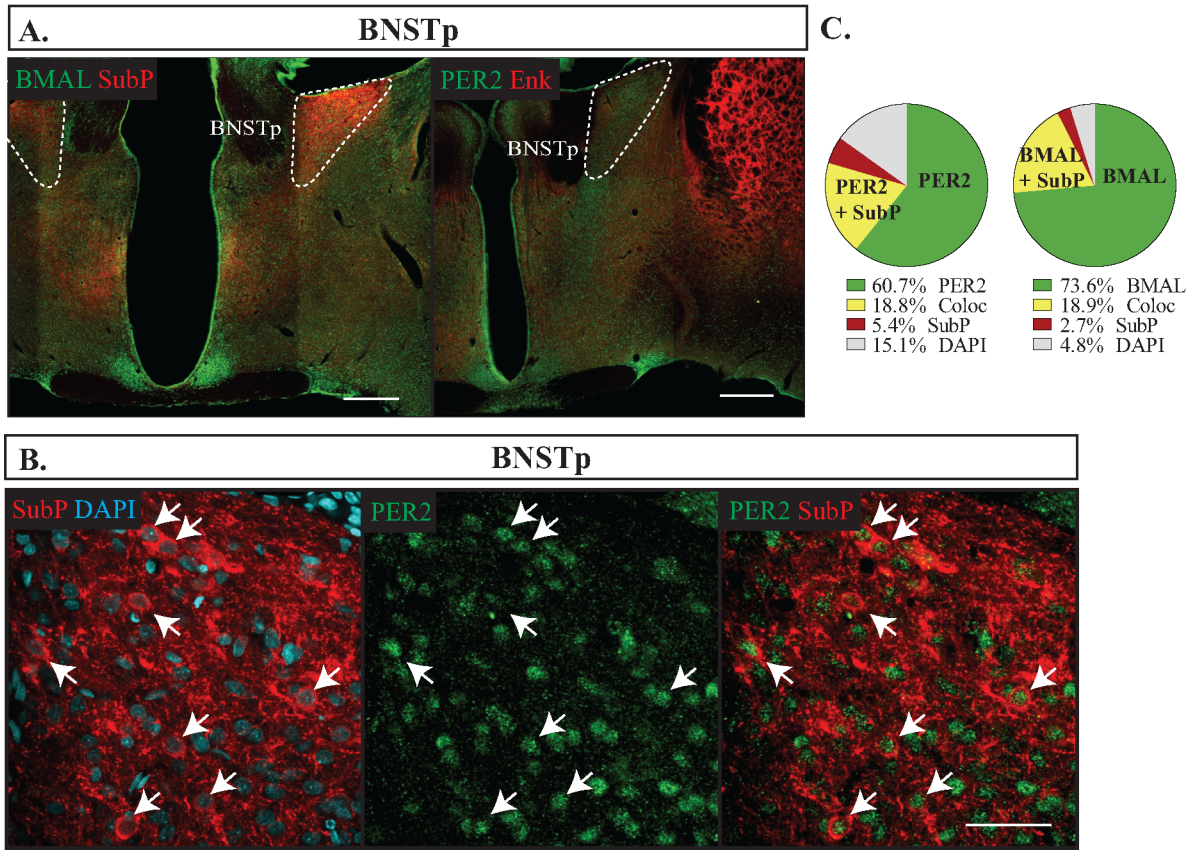

Figure 7. Localization of proteins in the BNSTp. (A) Outlines of regions imaged in the principal nucleus of the bed nucleus of the stria terminalis (BNSTp). The left panel is labelled with BMAL1 (green) and substance P (SubP) (red) and the right panel is labelled with PER2 (green) and enkephalin (Enk) (red). Individual confocal images were stitched together to create the single image. Scale bar: 500 µm. (B) Localization of proteins in the BNSTp showing PER2 or BMAL1 in green, Enk or SubP in red, and DAPI in blue. Arrows point to cells labelled with both proteins and indicate the same cell across a row. Scale bar: 50 µm. (C) Pie charts representing the proportion of each labelled cell combination in the BNSTp. Green: PER2 or BMAL1 with DAPI only, Yellow: PER2 or BMAL1 co-expressing SubP (Coloc), Red: SubP with DAPI only, Grey: cells identified with DAPI but not labelled with PER2, BMAL1, Enk or SubP.

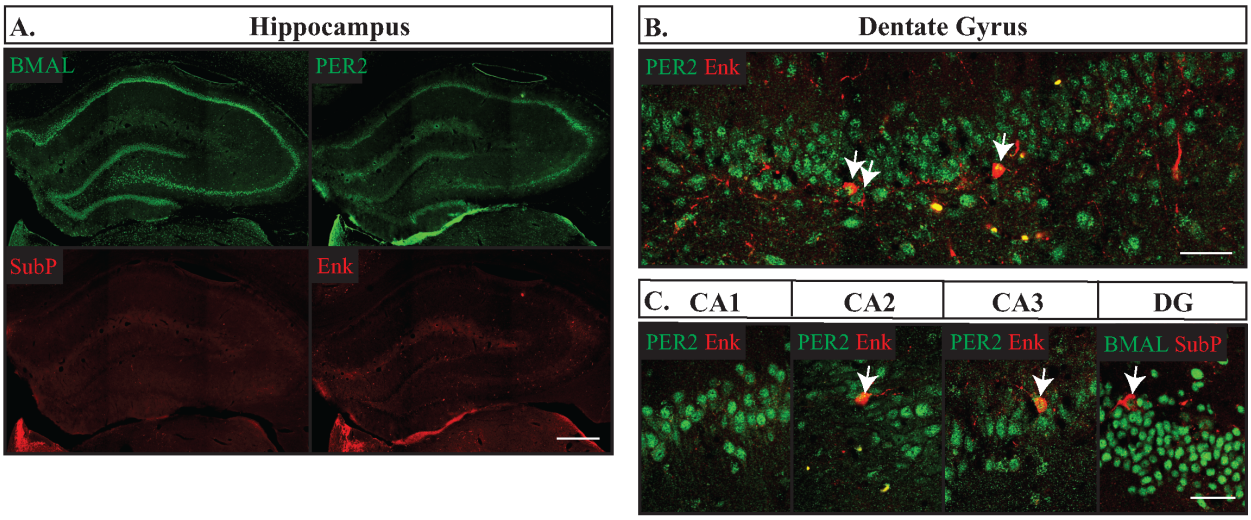

Figure 8. Localization of proteins in the hippocampus. (A) Localization of proteins in the hippocampus. Individual confocal images were stitched together to create the single image. Scale bar: 500 µm. Top left, BMAL1; top right, PER2; bottom left, Substance P (SubP); bottom right Enkephalin (Enk). (B) PER2 (green) and Enk (red) imaged in the dentate gyrus (DG) of the hippocampus. (C) PER2 or BMAL1 (green) and Enk or SubP (red) in regions CA1, CA2, CA3 and the dentate gyrus. Arrows point to cells labelled with both proteins. Scale bar: 50 µm.

Thalamic and hypothalamic nuclei (SCN, PVH, HabM, Arc)

As discussed above, the SCN, located in the medial hypothalamus, is perhaps the most important nuclei to the circadian system. This nucleus was only occasionally labelled with SubP or Enk (<1%) (Fig 9), as has been reported elsewhere (Welsh et al., 2010), and was therefore not analyzed further. A major target of the SCN is the hypothalamic paraventricular nucleus (PVH). The medial parvocellular region and the ventral region both had strong labelling with Enk, and some SubP, so are the regions analyzed here. Neurosecretory cells characterize this sub-region of the PVH, secreting regulatory hormones such as CRH and thyrotropin releasing hormone (TRH) to the median eminence where they act upon the anterior pituitary and regulate the neuroendocrine system (Ferguson et al., 2008). Another direct efferent target of the SCN is the arcuate nucleus in the hypothalamus (Arc), which regulates nutrient intake and energy balance. There are two populations of neurons in the Arc: 1) Neuropeptide Y and Agouti-related peptidic (NPY/AgRP) neurons associated with increased food intake and anabolism and 2) anorexic and catabolic related neurons that express pro-opiomelanocortin (POMC) (Joly-Amado et al., 2014). Both Enk and SubP are likely expressed in the NPY/AgRP set of neurons (Chronwall, 1985).

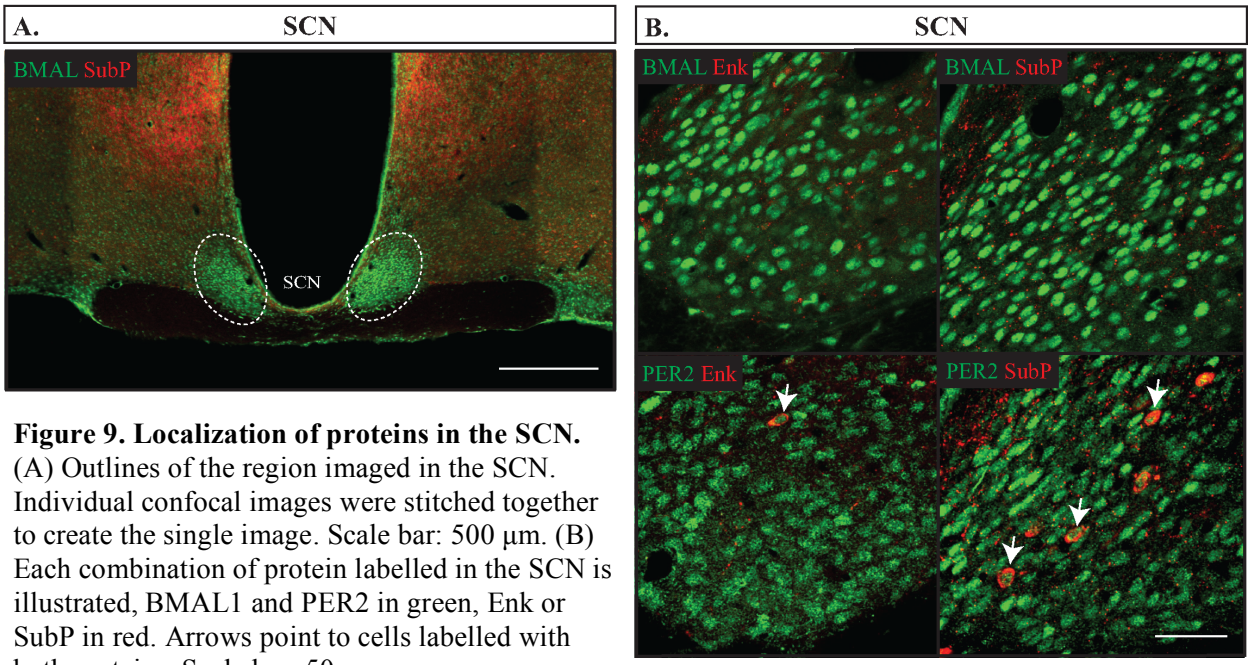

Figure 9. Localization of proteins in the SCN. (A) Outlines of the region imaged in the SCN. Individual confocal images were stitched together to create the single image. Scale bar: 500 µm. (B) Each combination of protein labelled in the SCN is illustrated, BMAL1 and PER2 in green, Enk or SubP in red. Arrows point to cells labelled with both proteins. Scale bar: 50 µm.

In each of these areas the variety of cell types and sizes was much more heterogeneous than the previously described regions. All DAPI labelled nuclei that were fairly large and round were included (for example, smaller irregularly shaped nuclei were excluded). BMAL1 and PER2 were well labelled in these cells, however at slightly lower proportions than in the limbic forebrain (varying from 60% to 80% of the total cells counted, as compared to around 90%) (Table 2). In the PVH, SubP was localized more in the ventral region and was almost always co-expressed with PER2 or BMAL1, whereas Enk was located throughout these regions and had a much higher proportion of cells labelled with Enk independent of BMAL1 and PER2. Co-expression with PER2 or BMAL1 occurred in only about two-thirds of the cells labelled with Enk (Fig 10). As has been reported elsewhere, SubP and Enk were relatively sparse in the Arc (Table 2) (Chronwall, 1985; Warden and Young, 1988), but this nucleus also had a higher proportion of cells labelled with Enk and not labelled with PER2 or BMAL1 (Fig 11).

The habenula is located in the dorsal thalamus and can be divided into two segments, the lateral habenula (HabL), which is often associated with limbic function, and the medial habenula (HabM), which is associated with motor and neuroendocrine function (Lecourtier and Kelly, 2007; Viswanath et al., 2013). The HabL was not labelled with Enk or SubP, and was therefore not examined. The dorsal region of the HabM is characterized by its high SubP and cholinergic expression (Lecourtier and Kelly, 2007). Of all the regions we analyzed, this region had the

highest levels of coexpression between SubP and BMAL1 or PER2, owing to more than 50% of its cells being identified with SubP, a proportion that is considerably higher than any other region we assessed (Fig 12). This region also continued to have high expression levels of PER2 and BMAL1 (Table 2)

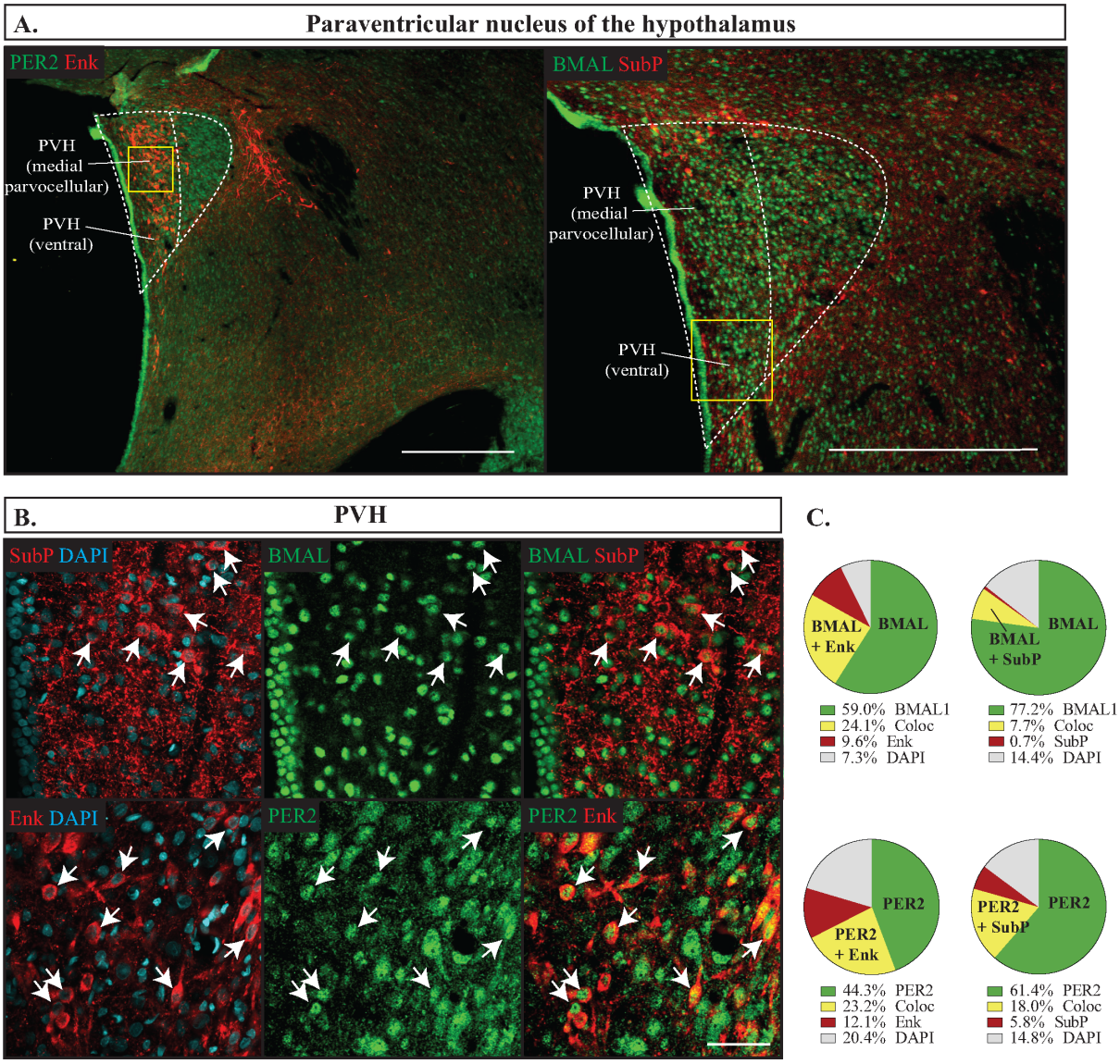

Figure 10. Localization of proteins in the paraventricular nucleus. (A) Outlines of regions imaged in the paraventricular nucleus of the hypothalamus (PVH). The left panel is labelled with PER2 (green) and Enkephalin (Enk) (red) and the right panel is labelled with BMAL1 (green) and Substance P (SubP) (red). Individual confocal images were stitched together to create the single image. Scale bar: 500 µm. The yellow box represents the region from where the below images were taken from. (B) Localization of proteins in the PVH. In the first panel Enk or SubP (red) is shown with DAPI (aqua). In the middle panel PER2 or BMAL1 (green). In the right panel BMAL1 or PER2 (green) are shown with Enk or SubP (red). Arrows point to cells labelled with both proteins and indicate the same cell across the row. Scale bar: 50 µm. (C) Pie charts representing the proportion of each labelled cell combination in the PVH. Green: PER2 or BMAL1 with DAPI only, Yellow: PER2 or BMAL1 co-expressing Enk or SubP (Coloc), Red: Enk or SubP with DAPI only, Grey: the remaining cells identified with DAPI but not labelled with PER2, BMAL1, Enk or SubP.

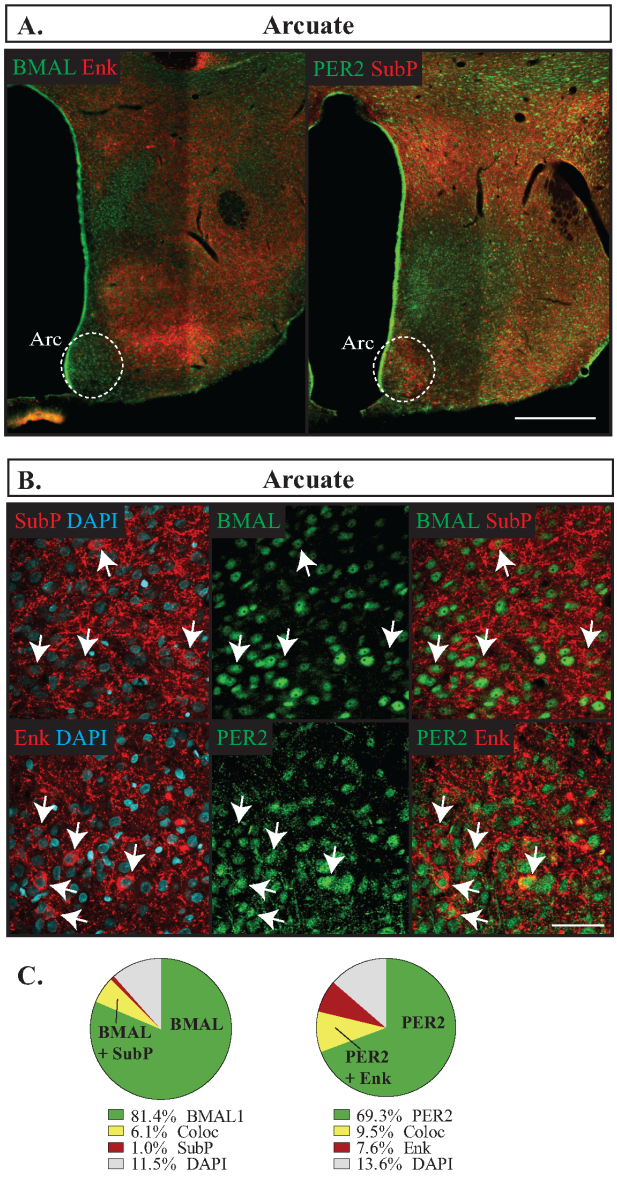

Figure 11. Localization of proteins in the arcuate nucleus. (A) Outlines of regions imaged in the arcuate nucleus (Arc). The left panel is labelled with BMAL1 (green) and Enkephalin (Enk)(red) and the right panel is labelled with PER2 (green) and Substance P (SubP)(red). Individual confocal images were stitched together to create the single images. Scale bar: 500 µm. (B) Localization of proteins in the Arc showing PER2 or BMAL1 in green, Enk or SubP in red, and DAPI in blue. Arrows point to cells labelled with both proteins and indicate the same cell across the row. Scale bar: 50 µm. (C) Pie charts representing the proportion of each labelled cell combination in the Arc. Green: PER2 or BMAL1 with DAPI only, Yellow: PER2 or BMAL1 co-expressing Enk or SubP (Coloc), Red: Enk or SubP with DAPI only, Grey: cells identified with DAPI but not labelled with PER2, BMAL1, Enk or SubP.

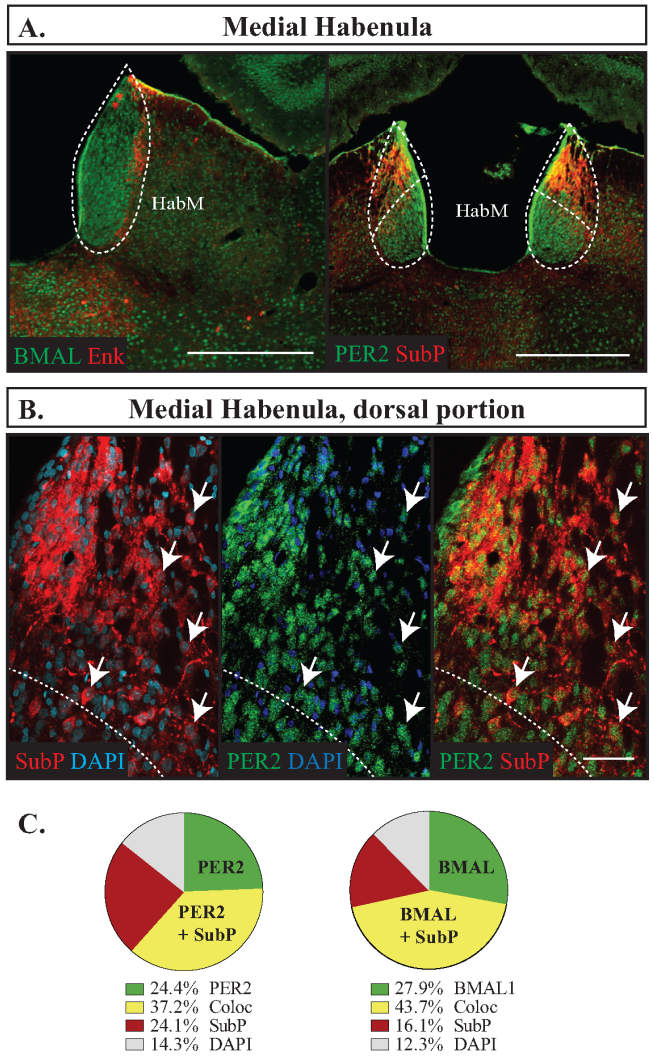

Figure 12. Localization of proteins in the medial habenula. (A) Outlines of regions imaged in the medial habenula (HabM). The left panel is labelled with BMAL1 (green) and Enkephalin (Enk)(red) and the right panel is labelled with PER2 (green) and Substance P (SubP)(red). Scale bar: 500 µm. (B) Localization of proteins in the dorsal portion on the HabM showing PER2 or BMAL1 in green, Enk or SubP in red, and DAPI in blue. Arrows point to cells labelled with both proteins and indicate the same cell across the row. Scale bar: 50 µm. Only the region above the dashed line were counted. (C) Pie charts representing the proportion of each labelled cell combination in the dorsal portion of the HabM. Green: PER2 or BMAL1 with DAPI only, Yellow: PER2 or BMAL1 co-expressing (Coloc), Red: SubP with DAPI only, Grey: cells identified with DAPI but not labelled with PER2, BMAL1, or SubP.

The Olfactory Bulb

Along with the retina, the olfactory bulb (OB) is the only neural structure that can autonomously sustain rhythmic clock gene rhythms (Abe et al., 2002). It also possesses its own local circuity and locally produced dopamine. Of all the areas we examined, the OB was unique in that it was the only brain region where BMAL1 and PER2 were not expressed homogenously throughout and was only found in a minority of cells, rather than the majority (Table 2). Furthermore, BMAL1 and PER2 also had different expression profiles. BMAL1 was highly expressed in the granule cell layer and moderately expressed in the glomerular area. Whereas PER2 was expressed in the mitral cells and some cells along the inner region of the glomerular layer (Fig 13a). Only Enk, and not SubP, was expressed throughout the OB. Enk densely labelled fibres in the granular layer, however cell bodies could only be identified in the glomerular layer, so this was the only region examined for co-expression. Unlike the previous brain regions mentioned, Enk, PER2 and BMAL1, for the most part, were expressed independently of each other (Fig 13b,c).

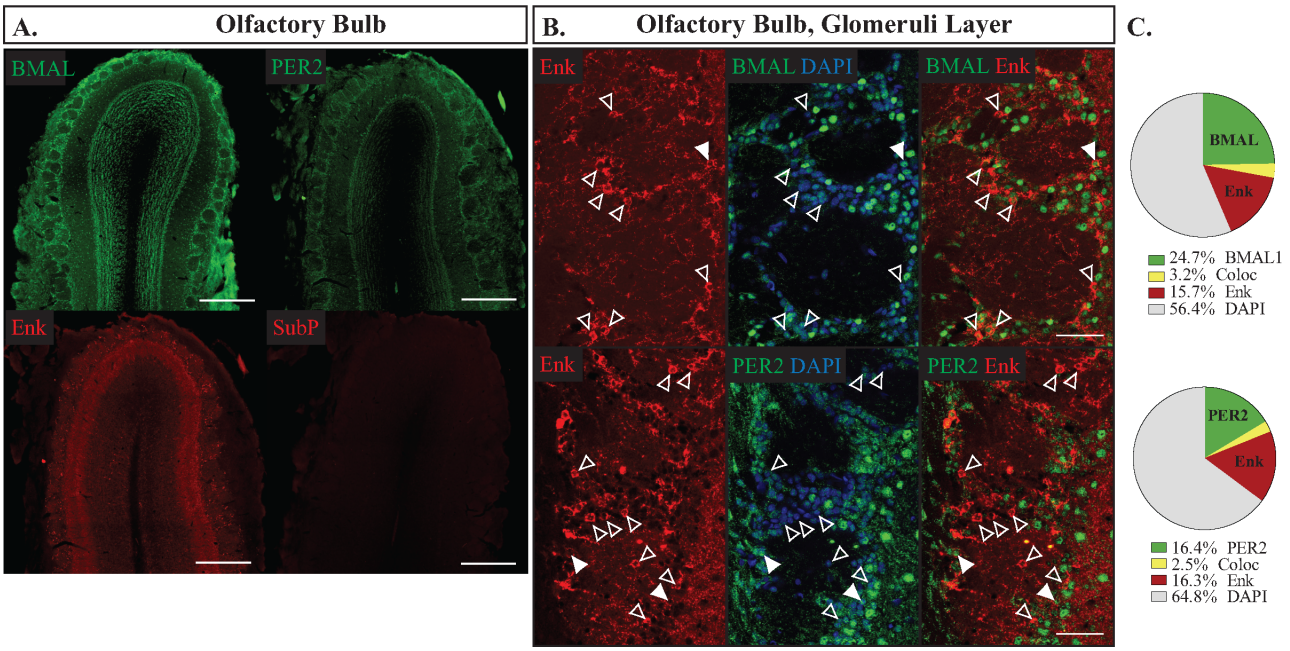

Figure 13. Localization of proteins in the olfactory bulb. (A) Localization of proteins in the olfactory bulb (OB). Single confocal images were stitched together to create the single image. Scale bar: 500 µm. Top left, BMAL1; top right, PER2; bottom left, Enkephalin (Enk); bottom right, Substance P (SubP). (B) Localization of proteins in the glomerular layer of the OB showing PER2 or BMAL1 in green, Enk or SubP in red, and DAPI in blue. Filled arrows point to cells labelled with both proteins, open arrows point to cells labelled with Enk only. Each arrow indicates the same cell across the row. Scale bar: 50 µm. (C) Pie charts representing the proportion of each labelled cell combination in the glomerular layer of the OB. Green: PER2 or BMAL1 with DAPI only, Yellow: PER2 or BMAL co-expressing Enk (Coloc), Red: Enk with DAPI only, Grey: cells identified with DAPI but not labelled with PER2, BMAL1 or Enk.

CONCLUSION

In this paper we characterized the coexpression of core circadian clock proteins with the neuropeptides SubP and Enk throughout the rodent forebrain. In most brain areas examined, PER2 and BMAL1 were homogenously expressed in the majority of neurons (~90%), so overall we cannot claim a co-expression preference for SubP or Enk, or any cell type for that matter. In most areas quantified, BMAL1 expression was higher than PER2, however this can be attributed to differences in the efficiency and clarity of the antibodies used. Both antibodies were tested in the SCN and demonstrated robust expression, indicating their specificity, however the PER2 antibodies produced high background, making distinctions of labelled cells more difficult in some areas. Two different PER2 antibodies were used, both producing similar staining patterns, with the exception of background levels. The antibody that produced the least amount of background was chosen for each region in order improve accuracy in counting.

SubP and Enk densely labelled fibres, so colchicine was injected into the ventricles 24 h prior to brain removal to improve cell body labelling. This improved visualization of cell bodies, however areas such as the dorsal striatum and nucleus accumbens still remained quite noisy, and we estimate that the actual proportion of cells expressing either SubP or Enk to be higher than reported here. For example, in the dorsal striatum, it has been reported that cells expressing SubP or Enk are close to 50% (Gerfen and Young, 1988; Gangarossa et al., 2013b). Here, we report numbers closer to 30-40% (Table 1). Nonetheless, the distributions were similar, and cells expressing both SubP or Enk were clearly observable in cells expressing PER2 and BMAL1. In addition, since SubP and Enk are nearly equally distributed throughout the dorsal striatum and since PER2 and BMAL1 were expressed in the majority of cells, we can confidently conclude that these clock proteins are expressed in both cell types. In fact, we can conclude that throughout the brain, it is likely that any region that has been shown to have rhythmic clock gene expression is expressing those genes ubiquitously in all neurons.

On the other hand, the hypothalamus and the OB showed lower expression levels of BMAL1 and PER2 so may be an exception to the ubiquitous expression of these proteins. While this is likely the case in the OB, where the expression levels were remarkably lower, the nuclei in

the hypothalamus have highly heterogeneous cell types and without a marker for neuronal cell types, we cannot ascertain that the populations we counted completely excluded glia and other non-neural cell types. Regarding this issue, further exploration in the thalamus and hypothalamus is warranted.

DISCUSSION

Enkephalin and Substance P as a marker for D1- and D2- receptors

The study that motivated this project demonstrated that there were functional differences between D1- and D2-class dopamine receptors in entraining clock genes in the dorsal striatum (Hood et al., 2010). Clock gene rhythms are impaired in dopamine-depleted brain regions (Hood et al., 2010; Gravotta et al., 2011), and Hood at al. concluded that this was due to loss of activity of the D2-receptors, as no effects were seen when D1-receptors were independently blocked. Similarly, once dopamine-depleted, D2-receptor activation was able to re-entrain clock gene rhythms, while D1-receptor activation was not. This led to the question of how clock genes are expressed in the central nervous system outside of the the SCN, and if they are preferentially expressed in different cell types. SubP and Enk were chosen as they are preferentially expressed in D1- and D2-receptor bearing neurons in the striatum, but they also represent a variety of different subpopulations of cells in other brain regions (Gray et al., 1984; Chronwall, 1985; Everitt et al., 1986; Gerfen and Young, 1988; Warden and Young, 1988; Lu et al., 1998).

We have shown here that the functional differences between the ability of D1- and D2-receptors to entrain clock genes are not due to differences in these cells' ability to express clock genes. This may instead be due to their signaling mechanisms. Considering the opposite intracellular effects of D1- and D2-receptors (Tritsch and Sabatini, 2012), Hood et al. may not have examined the correct conditions to properly implicate D1-receptors in clock gene entrainment. This is supported by a recent study by Gallardo et al. (2014) who showed that D1-receptor knock-out impairs *Per2* rhythms in the dorsal striatum, and that in intact rats, daily injections of a D1-receptor agonist was able to cause behavioural entrainment. Although they did not examine clock gene rhythms with the behavioral entrainment, this indicates that D1-receptors can contribute to aspects of circadian rhythmicity. The interactions between these dopamine

circuits will need to be further studied, taking care of the experimental conditions used targeting both D1- and D2- receptor activation and deactivation patterns in order to better understand signals that are important for clock gene entrainment in the dorsal striatum.

Entraining rhythms downstream of the SCN

With the exception of the SCN, there is still very little known about clock genes in the central nervous system, including which signals are important for their entrainment, how they contribute to behaviour and what happens when their timing is diminished or out of phase. The SCN, retina and OB are the only known tissues in the body that are able to fully maintain autonomous rhythms in isolation from other tissues and entrainment signals (Abe et al., 2002). All other downstream tissues are known as "subordinate oscillators," relying on the SCN and other oscillating signals to produce rhythmic clock-gene expression. Both neural connections and secreted factors are important for SCN output (Dibner et al., 2010; Welsh et al., 2010). In the brain this neural output pathway is mostly multi-synaptic (Guilding and Piggins, 2007), in a system that is already overwhelmingly complex, making it even more complicated to understand exactly which input signals are required for rhythm generation in each brain region.

It is likely that direct neural connections from the SCN entrains rhythms of the major output regions of the SCN. The subparaventricular zone (SPZ) receives the greatest number of efferents from the SCN and is involved in many aspects of circadian rhythmicity, including body temperature, activity rhythms and contributes to the sleep-wake cycle, potentially acting as a site that determines a species as diurnal or nocturnal (Schwartz et al., 2004; Guilding and Piggins, 2007; Ramanathan et al., 2010; Schwartz et al., 2011; Morin, 2013). The PVH also receives dense projections from the SCN and is considered to be important for timing in the neuroendocrine system (Guilding and Piggins, 2007; Bechtold and Loudon, 2013). Most of the SCN projections to the PVH synapse with the parvocellular neurons, which are neurosecretory and project to the median eminence, releasing regulatory hormones that target the anterior pituitary (Ferguson et al., 2008). The direct SCN-PVH connections are likely important to the timing of activating major hormonal pathways such as the stress-axis and the thyroid-axis, among others. Neural activity, influenced by the retino-hypothalamic inputs, is important to entraining clock gene rhythms in the SCN (Colwell, 2011). Likewise, it is highly probable that SCN inputs are driving rhythms in the SPZ and SCN. The neural activity in these areas are

inverse from the SCN and directly inhibited by SCN inputs (Nakamura et al., 2008). They also display similar entrainment patterns in electrical activity as the SCN following phase shifts in lighting schedules.

Other regions, receiving only sparse projections from the SCN have also been shown to directly depend on SCN connections for rhythm production. These include the habenula, Arc and BNSTov. The habenula receives widespread inputs from limbic areas such as the prefrontal cortex, nucleus accumbens and BNST, plus other areas of the brain, including the Arc and locus coeruleus (Viswanath et al., 2013). The main output of the medial-dorsal region of the HbM, the SubP expressing region examined here, is to the interpeduncular nucleus in the midbrain, a relay nucleus that influences many dopaminergic centres in the brain, and linked with a broad array of functions such as sleep, stress response, and nicotine withdrawal (Lecourtier and Kelly, 2007). Many neurons in the habenula show a diurnal variation in resting membrane potential and firing rate that is dependent on functional clock proteins (Sakhi et al., 2014). Though not much is understood of how the habenula influences circadian rhythms, lesioning the output of this nucleus alters daily locomotor activity patterns and increases the free-running period in constant darkness (Paul et al., 2011).

The Arc has a strong influence on nutrient intake and energy balance and is located in close proximity to the median eminence where it receives information from ghrelin, leptin and insulin circulating in the blood (Joly-Amado et al., 2014). The communication between the Arc and the SCN is bidirectional, as the Arc projects to the SCN as well as other regions of the hypothalamus, including the PVH, and forebrain regions such as the VTA (Morin, 2013). Thus, the Arc has the ability to influence both neuroendocrine and autonomic output but also higher order processing such as affect and motivation. The Arc expresses robust rhythms in gene expression and is one of the few brain regions outside of the SCN and OB that can maintain *Per2* oscillations in-vitro over multiple days (Abe et al., 2002). Arc lesions have been found to prevent pre-prandial increases in body temperature in anticipation of timed meals (Wiater et al., 2013), thus, the Arc likely contributes to circadian rhythmicity by influencing timing of the autonomic system and interacting with the PVH in timing of the neuroendocrine system, and coordinating peripheral functions such as cardiac function, thermogenesis and metabolism with nutrient intake.

The BNST plays a role in stress and anxiety-related behaviours and is closely associated with the CEA (Alheid, 2003). Factors influencing their entrainment are discussed more extensively below, however the BNST is one of the few limbic centres that are connected monosynaptically with the SCN (Morin, 2013). Not only have tracing studies demonstrated the direct link between the SCN and BNST, but unilateral SCN lesions have been found to diminish rhythmic PER2 expression on the ipsilateral side (Amir et al., 2004). Similar results have also been found in the habenula, with an ipsilateral decrease in *cFos* rhythms with unilateral SCN lesions (Guzman-Ruiz et al., 2014), suggesting a functional role of the SCN connections in rhythm production. However, these areas receive rhythmic inputs from other sources that could also contribute to rhythmicity, such as the circulating hormonal factors related to feeding that regulate neural activity in the Arc (Joly-Amado et al., 2014). Similarly, the habenula receives direct inputs from the retina, and many of the neurons here are light responsive (Zhao and Rusak, 2005). Both of these sources of inputs oscillate on a daily scale and could provide signals that contribute to clock-gene rhythms.

Ever since rhythmic clock gene expression was found in the striatum and limbic forebrain (including the NAc, Tub, BLA, CEA, BNSTov, BNSTp and Hip), the factors that drive their rhythms have become a topic of interest. These structures are generally not associated with circadian function and, for the most part, are not directly connected with the SCN, so it is likely that these areas are entrained by more remote signals. Together, the striatum and limbic forebrain receive dopamine modulation from the VTA and regulate our emotional processing, including mood, affect, motivation, memory, anxiety, and stress and fear responses; all of which are varied in a diurnal manner as an animal goes through its sleep-wake cycles (Chaudhury et al., 2005; Webb et al., 2009; Smarr et al., 2014; Webb et al., 2015). So we can postulate that the circadian system drives these variations and contributes to the more subtle aspects of behaviour that drive locomotor activity, feeding, substance abuse and learning. These activities could also provide entrainment signals, since all of these behaviours also alter the neural activity of these structures, thus greatly increasing the number of potential entrainment signals.

The striatum and many of the limbic structures have been shown to rely on an intact SCN for rhythmic dopamine function and rhythmic clock gene production (Iijima et al., 2002; Sleipness et al., 2007). However, this only demonstrates that these structures are not autonomous oscillators since SCN ablation results in complete loss of rhythms, rendering behaviour, hormone

secretion, eating patterns and sleep-wake patterns completely arrhythmic. A number of studies that have been able to dissociate the timing of clock-gene rhythms from their "natural" phases with the SCN provide more valuable information in elucidating the types of signals that can contribute to entrainment. As mentioned above, loss of dopamine diminishes clock-gene rhythms in the dorsal striatum, BNSTov and PVH (Hood et al., 2010; Gravotta et al., 2011), and daily injections of a D2-dopamine receptor agonist is able to entrain PER2 rhythms to a new phase, independent of the SCN (Imbesi et al., 2009; Hood et al., 2010). Likely related to a similar pathway (Okada et al., 1996), knockout of the adenosine transporter ENT1 also diminishes PER2 rhythms in the nucleus accumbens, with daily injections of an A_{2A}-adenosine receptor agonist being able to restore these rhythms, or shift them in wild-type mice (Ruby et al., 2014). Extracellular dopamine levels and many other dopaminergic functions are rhythmic in the striatum making dopamine a likely candidate for indirect entrainment, but dopaminergic neurons are not pacemakers on their own and must be entrained from another source (Paulson and Robinson, 1994; Castaneda et al., 2004; Hood et al., 2010; Ferris et al., 2014). A multi-synaptic neural pathway from the SCN is probable, with more than one SCN outputs acting as possible relays. An indirect route could be from the habenula, with the lateral habenula projecting to the striatum and VTA and and the medial habenula projecting to the interpeduncular nucleus, which then also projects to the VTA (Lecourtier and Kelly, 2007; Viswanath et al., 2013). Another major output of the SCN is the paraventricular nucleus of the thalamus (PVT) (Colavito et al., 2015; Kirouac, 2015). This midline nucleus also receives afferents from the the Arc and PVH (also directly connected with the SCN) and projects considerably throughout the limbic forebrain, making it a likely site communicating timed daily feeding and energy homeostasis information to motivation and arousal-related centres.

Timed daily access to food (also known as timed restricted feeding) and chronic methamphetamine administration offer some interesting insights into non-photic and SCN-independent circadian entrainment mechanisms. Both of these protocols induce daily anticipatory locomotor activity and can restore behavioural and clock-gene rhythms in completely arrhythmic rodents following SCN ablation or under constant light (Honma et al., 1987; Honma et al., 1992; Masubuchi et al., 2000; Lamont et al., 2005; Waddington Lamont et al., 2007; Mistlberger, 2009; Mohawk et al., 2013; Natsubori et al., 2014). Under daytime restricted feeding, anticipatory locomotor activity peaks several hours prior to the expected

mealtime, at a time when the rodent is usually sleeping. This also alters the phase of PER2 rhythms in the dorsal striatum, substantia nigra, BLA and hippocampus, nearly inversing the rhythms and synchronizing the peak expression time to be around ZT17, putting them in phase with the SCN (Verwey et al., 2007; Waddington Lamont et al., 2007; Segall et al., 2008; Natsubori et al., 2013a). Interestingly, when feeding is switched to nighttime, the phase of PER2 rhythms in these limbic structures is unaffected, but the BNSTov and CEA inverse and synchronize with the BLA and hippocampus, without affecting the SCN (Verwey et al., 2008). Similar phase changes are also experienced with chronic methamphetamine, and if locomotor activity is completely transposed to the light phase, this is followed by flipped PER2 rhythms, as seen with daytime restricted feeding (Masubuchi et al., 2000; Natsubori et al., 2013b; a; 2014). The mechanisms behind the food entrainable oscillator and the methamphetamine entrainable oscillator are not understood (Mistlberger, 2009; Smit et al., 2013), however both have been linked to dopamine function in the limbic forebrain. For restricted feeding, targeted lesions throughout the brain, including the limbic forebrain have not been able to locate a discrete brain area as the "clock centre", unlike the SCN, which acts as the centre for the light entrainable oscillator (Davidson, 2009). This points to a "systems approach", and the dopamine system may be a contributor as D1-dopamine receptor knockout has been able to prevent entrainment to restricted feeding, with a targeted knock-in of dopamine function in the dorsal striatum being sufficient to restore anticipatory behaviour (Gallardo et al., 2014). Methamphetamine is known to act upon D1- and D2-receptors, as well as NMDA receptors, and dopamine antagonists are able to shift the phase of methamphetamine entrained locomotor activity (Honma and Honma, 1995; Shibata et al., 1995; Mohawk et al., 2013). From these studies we can conclude that if the same principle exists here as in the SCN, and that rhythmic neural activity is enough to drive clock gene rhythms, then feeding and drugs of abuse would provide strong enough incentives to activate the arousal and reward-related pathways, eliciting sufficient neural changes in these pathways to entrain clock genes that correspond to behavioural changes.

Hormones can also play an important role in clock-gene entrainment, and may be playing a more important role than direct SCN inputs in the BNSTov and CEA. Both glucocorticoid and thyroid hormones are important to maintaining the normal PER2 rhythms in the BNSTov and CEA. Removal of these hormones by adrenalectomy or thyroidectomy, respectively, blunts peak expression in these regions (Amir et al., 2004; Amir and Robinson, 2006). Though the concept

has not been explored in thyriodectomized animals, timed daily injections of glucocorticoids re-entrains PER2 rhythms independently from the SCN, paralleling the function of striatal dopamine in these structures (Segall and Amir, 2010). The proposed pathway of glucocorticoid regulation on clock-genes is quite direct. *Per1* and *Per2* both have glucocorticoid response elements in their promoter region (Yamamoto et al., 2005; So et al., 2009). Since both the CEA and BNSTov express glucocorticoid receptors, clock-genes in these nuclei should be regulated by the normal daily rise and fall of corticosterone along with acute changes that occur with stressful events (Tahara et al., 2015). Intriguingly, restricted feeding seems to be able to override glucocorticoid signaling, and can re-entrain PER2 rhythms in adrenalectomized rats (Segall et al., 2008), again, independently from a fully functioning SCN. To further add to the complexity, sex hormones may also play a role in clock-gene entrainment. In cycling female rats, PER2 amplitudes vary with the phase of their estrous cycle and continuous estrogen replacement causes a decrease in peak expression levels (Perrin et al., 2006).

On the other hand, the CEA and BNSTov not only respond to stress hormones, such as corticosterone, but may be involved in modulating stress-responses along with the PVH, since these nuclei also possess CRH-producing neurons (Day et al., 1999; Daniel and Rainnie, 2016). In preliminary studies using double stranded RNA interference targeted for CRH mRNA in the BNSTov, gene silencing decreased PER2 expression locally (Amir and Stewart, 2009). Preliminary results from this study suggested that PER2 and CRH were not expressed in the same cells, so the authors concluded that the effects were indirect. With the immunohistochemical techniques they used, it is clear that there are cells expressing PER2 and not CRH, but it is difficult to confirm that co-expression does not occur. CRH is estimated to be expressed in approximately 25 % of GABAergic cells in this region (Day et al., 1999) and with the present findings that PER2 is expressed in nearly all neurons, it is more likely that co-expression between CRH and PER2 does occur here, so the entrainment effects may be both direct and indirect.

Another important aspect to maintaining rhythm amplitudes and producing a coordinated output that properly communicates circadian information, whatever it might be, is the ability to maintain phase synchrony between cells within a brain region, and between brain regions interacting toward a common function. It is possible that in these heterogeneous brain regions, one cell type is used to generate intracellular rhythms and another is used to maintain phase

synchrony between cells. This is a concept that has been studied extensively in the SCN, and is still poorly understood, so it is not surprising that it has never been looked at in downstream regions. In the SCN, there is a very heterogeneous mixture of cell types expressing a variety of different neuropeptides (Welsh et al., 2010). Furthermore, the SCN can maintain robust autonomous rhythms in electrical activity and clock-gene expression for months at a time in tissue culture, yet only about 60% of individual neurons will maintain rhythms if cultured at low density; and these rhythms will all possess independent phases and period lengths (Welsh et al., 2010; Brancaccio et al., 2014). The working hypothesis by many scientists is that specific cell types in the SCN support high amplitude rhythms (such as argenine vasopressin peptide) and others couple cells within the circuit to synchronize and strengthen the rhythms at a tissue level (such as vasoactive intestinal peptide) (Aton et al., 2005; Maywood et al., 2006; Mohawk and Takahashi, 2011; Brancaccio et al., 2014). Intercellular network communication seems to be key to maintaining a fully functional SCN and if synaptic activity is blocked, rhythmicity breaks down. Promoters on the negative arm of the circadian transcription-translation feedback loop (on the *Per* and *Cry* genes) are responsive to cAMP response element-binding protein (CREB), so the link between electrical activity and clock-gene expression is likely the intracellular calcium and CRE-dependent signaling cascades (Welsh et al., 2010; Brancaccio et al., 2014). I propose that entrainment in downstream regions is similar to the SCN, where rhythms generated in one cell type are important for entraining rhythms in the surrounding cell types through their intercellular network communication pathways, However, in the SCN the original rhythm generators may be specific to certain cell types, whereas in downstream regions the cell type that generates the initial rhythm may be more flexible, allowing circadian entrainment to follow the more potent inputs.

In other brain regions, the circuit organization has evolved for purposes other than driving circadian rhythms, so it is not surprising that they lack robust, self-generating rhythms. The evolutionary advantage of this is that it provides a more flexible system that is able to adjust behaviours with shifts in environmental factors. Yet the importance of network communication is likely just as important. An example is the dorsal striatum, a structure that shifts its timing of clock-gene expression with the availability of food (Natsubori et al., 2013a). This is likely due to changes in electrical activity, including dopaminergic signaling. Specific D1- or D2-receptor signaling may activate intracellular signaling pathways that feed into the transcription-translation

feedback loop. However, neuromodulators such as dopamine not only alter each individual cell's electrical activity but at a network level, it alters intercellular coupling and communication with its afferent and efferent regions (Ruskin et al., 1999a; Sharott et al., 2005; Gatev et al., 2006; Lemaire et al., 2012). With this model, an initial change in an environmental factor can produce a behavioural response, that if repeated, causes repeated changes to the entire cellular network's electrical activity that will feed into the circadian genes in that region, altering their phase and expression patterns. It is likely that if any element of the network circuitry breaks down it will impair the original basic functioning of that nucleus and the circadian expression of its clock-genes, whether it be D1- or D2-receptor functioning in the dorsal striatum, or CRH-production or glucocorticoid removal in the BNSTov. It is thus likely that if one cell type is impaired the others will be too.

The olfactory bulb as a unique model for studying circadian entrainment

The OB may provide interesting insights to the circadian system, as it seems to be unique in its functioning. Similar to the SCN, its rhythms are autonomously generated, yet its primary role is considered to be for processing olfaction. Unlike most other structures studied here, it does not produce robust clock gene rhythms homogenously throughout (Granados-Fuentes et al., 2004). Where the SCN entrains primarily to light, the primary zeitgeber (environmental time cue) in the OB is not understood; although locally produced vasoactive intestinal peptide may contribute to local rhythm production (Miller et al., 2014). It receives phase information from the SCN, yet it will continue to oscillate without SCN input, in behaviourally arrhythmic animals and in isolated tissue cultures (Abe et al., 2002; Granados-Fuentes et al., 2004). Circadian output influences olfactory responsivity (Amir et al., 1999; Granados-Fuentes et al., 2006), but despite extensive connectivity to limbic centres and a potential role in complex behaviours in rodents (Slotnick, 2001), no one has yet been able to link its removal to circadian behaviours such as locomotor activity patterns or food anticipatory effects (Davidson et al., 2001). Its accessibility as compared with other brain structures, plus its locally produced dopamine and local circuitry makes it an interesting brain region to study, and perhaps uncovering mechanisms here may help to understand circadian function in other, non-SCN brain regions.

Summary

The results found here indicate that clock-gene expression does not seem to be limited to any specific cell type within the brain. By extending what is known about clock-gene entrainment in the SCN to downstream regions, we propose that the entire network circuitry, and not just one cell type, is important for maintaining coherent clock-gene rhythms. Further research exploring how targeted neuronal activation and cell-cell communication will be important to answering these questions. Further developments from this research may be able to help answer questions including how clock-gene output from regions such as the limbic forebrain interact with behaviour, and how disruptions to clock-genes in these regions affect the organism as a whole.

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